Promoter Analysis and Endosperm-specific Expression of Rice Phytoene Synthase Genes (*psy1* and *psy2*) in Rice

LEUNG, Chiu Yi

A Thesis Submitted in Partial fulfillment of

the Requirement for the degree of

Master of Philosophy

in

Molecular Biotechnology

The Chinese University of Hong Kong

June 2011


香港中文大學
圖書館系統
14 AUG 2012
LIBRARY SYSTEM
THE CHINESE UNIVERSITY OF HONG KONG

Thesis/ Assessment Committee

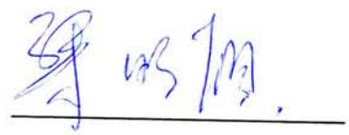
Professor Lam, H. M. (Chair)

Professor Sun, S. S. M. (Thesis Supervisor)

Professor Jiang, L. W. (Committee Member)

Statement

All the experimental work reported in this thesis was performed by the author, unless specially stated otherwise in the text.



LEUNG, Chiu Yi

Acknowledgements

First of all, I would like to express my sincere gratitude and respect to my supervisor, Prof. S. S. M. Sun, for offering me such an invaluable opportunity to experience the life of being a scientist, a dream since I was small; and to work in the field of plant biotechnology under his guidance and patience throughout my study. I am grateful for his precious advice, concern and criticisms on my research and broadening my horizons by encouraging me to participate in overseas summer course, conference and competition. These invaluable experience helped building up my confidence.

My gratitude must also go to the members of thesis committee, Prof. H. M. Lam, Prof. L. W. Jiang and Prof. W. K. Yip, for their precious suggestions and comments on my work; and also for reviewing my thesis.

I would like to express my truly appreciation to Angela Yu, Ken Lau and Marilyn Yu for their technical supports and efficient works, allowing me to work out my experiments smoothly. Heartfelt thanks must go to Angela Yu for her sincere and supportive emotional backup.

Endless thanks must also go Jennifer Wai, Anthony Chan and Eric Suen for their unlimited support and encouragement when I faced setbacks and were hesitated to

step forward; Pauline Yip and William Ho for their technical support in LC analysis. Also special thanks must go to all my labmates, Steffi Long, Jianny Wang, Flora Tian, Jim Yang, Veronica Yu, Joun Joun Lee, Gundam Wong, Ringo Kowk, Ronald Yu, Sylvia Lok and Raymond Cheung not only for their technical support and advice on my project, but also for giving me such a happy and unforgettable memory in my life. I am really glad to have the chance to work, to share and to laugh with them in G90.

I would like to thank Bondy Lai, Mr. Nagi and K. H. Man for assisting me to keep plants in green house and Gene Garden; and all members of AoE, staffs from Team A and Team C of the Biology Department for their administrative work and technical support, especially Freedie Kwok for assisting me in sample preparation and the use of fluorescence microscope.

Finally, I would like to show my deepest thanks to Louisa Lam, my family, and my friends, Kiwi Lam, Melody San and Crystal Lee, for sharing endless support, laughter and love with me in this period of time; especially to Davyn Wan, for always standing by me during the difficult time in my graduate study, whose patience and love encouraged me to move forward and complete this work.

Abstract

Vitamin A deficiency (VAD) is a global health issue affecting more than 26 developing countries and millions of children worldwide. In order to combat VAD, the Golden Rice project was launched which made use of the phytoene synthase (*psy*) from maize and a bacterial desaturase (CrtI) to boost the β-carotene content up to 37μg/g of rice endosperm. In this project, we focused on the characterization of rice's own *psy* genes, and their potential to enhance β-carotene content in rice endosperm. Early studies have identified three rice *psy* homologs in the genome and were shown to have differential expression patterns. While OsPSY3 was proposed to be related to abiotic stress-induced abscisic acid formation, the roles of OsPSY1 and OsPSY2 have not been well characterized. We investigated the tissue-specific activity of *Ospsy1* and *Ospsy2* by constitutively expressing them under the control of *CaMV35S* promoter in rice. With different preferences in membrane association, *Ospsy1* and *Ospsy2* would be active in different tissues. Such differences would be determined by monitoring the changes of carotenoid level in different tissues.

Constitutive expression of *Ospsy1* led to a boost in β-carotene up to 3190ng/mg in dry rice calli, but to a severe carotenoids reduction in leaves due to gene silencing; while constitutive expression of *Ospsy2* increased β-carotene up to 1796ng/mg of dry

calli, and up to 43016ng/mg of dry leaves. On the other hand, rice seed-specific Gt-1 promoter was used to drive the expression of *Ospsy1* and *Ospsy2* in order to enhance the phytoene content in rice seeds. Phytoene enhancements averaging 145.6% to 34.4ng/mg and 41.9% to 19.87ng/mg of dry rice endosperm were observed for *Ospsy1* and *Ospsy2* respectively. These confirmed the seed-specific activity of *Ospsy1* and its potential use as a candidate for developing Golden Rice.

Furthermore, seven endosperm specific GCN4 motifs (TGAGTCA) were inserted into the leaf-specific *Ospsy1* promoter to introduce seed-specific activity. The activity of the modified promoter was determined using beta-glucuronidase (GUS) reporter gene. GUS histochemical staining and activity assay revealed that the introduction of GCN4 multimer to the leaf-specific *Ospsy1* promoter, which is absent in the native copy, can drive GUS expression in rice endosperm.

摘要

維生素 A 缺乏是全球性健康問題之一。全球超過 26 個發展中國家及數百萬兒童正受維生素 A 缺乏的威脅。為了減低維生素 A 缺乏引致的問題，Dr. Potrykus 的團隊致力將類胡蘿蔔素的生物合成過程轉化進稻米中，並於 2005 年，成功轉化玉米八氫番茄紅素合成酶(*psy*)及細菌脫氫酶(CrtI)致稻米種子，提升水稻胚乳類胡蘿蔔素含量高達每克穀物 37 微克。在本研究中，我們針對水稻本身的八氫番茄紅素合成酶基因進行分析，並測試它們在提高水稻胚乳類胡蘿蔔素的潛力。研究發現水稻基因組中共有三個八氫番茄紅素合成酶基因，表達在水稻不同的組織。當中除 OsPSY3 有助植物抗逆外， OsPSY1 及 OsPSY2 的功能仍未有詳細分析。有見及此，我們以持續性表現 *CaMV35S* 的啟動子將 *Ospsy1* 和 *Ospsy2* 表達在不同水稻組織中，並對它們的活性進行分析，從而了解它們在不同組織的差異。

在持續性表現啟動子的影響下，*Ospsy1* 提升水稻癒傷組織類胡蘿蔔素含量至每微克 3190 納克；但葉片中的類胡蘿蔔素卻嚴重下降。相反，*Ospsy2* 不但增加水稻癒傷組織類胡蘿蔔素含量至每微克 1796 納克，更提升葉片類胡蘿蔔素含量高達每微克 43016 納克。另一方面，在水稻種子特異啟動子(Gt-1)的驅動下，*Ospsy1* 和 *Ospsy2* 分別提高水稻種子中八氫番茄紅素含量平均高達 145.6%至每微克穀物 34.4 納克和 41.9%至每微克穀物 19.9 納克。這證實了 *Ospsy1* 的種子特

異活性及其用以製作黃金稻的潛力。

此外，為了使 *Ospsy1* 在種子中表達，我們在其啟動子加入 7 個 GCN4（TGAGTCA）水稻胚乳特異調節因子，連接 GUS 報告基因，利用轉基因水稻測試它的種子特異性。GUS 組織染色和活性測定顯示，加入 GCN4 調節因子後，*Ospsy1* 啟動子成功驅動 GUS 基因在水稻胚乳表達，使它具水稻種子特異性。

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AA	:	amino acids
ABA	:	abscisic acid
ABRE	:	ABA-responsive element
AS	:	acetosyrigone
ATP	:	adenosine triphosphate
bp	:	base pair
BSA	:	bovine serum albumin
CaMV35S$_{pro}$	:	cauliflower mosaic virus 35S promoter
CCDs	:	carotenoid-cleaving dioxygenases
cDNA	:	complementary DNA
CHY-b	:	ε-hydroxylase
CHY-e	:	β-ring hydroxylase
CrtB	:	bacterial phytoene synthase
CrtI	:	bacterial carotene desaturase
CrtISO	:	carotene isomerase
CTAB	:	cetyltrimethylammonium bromide
DAF	:	day after flowering
DEPC	:	diethylpyrocarbonate
DIG	:	digoxigenin
DNA	:	deoxyribonucleic acid
dNTPs	:	deoxyribonucleotide triphosphates
DTT	:	dithiothreitol
EDTA	:	ethylenediaminetetra-acetic acid
EMV	:	empty vector
FAD	:	flavin adenine dinucleotide
GGPP	:	geranylgeranyl diphosphate
Gt-1$_{pro}$	:	rice glutelin-1 promoter
GR	:	Golden Rice
GUS	:	β-glucuronidase
HRP	:	horseradish peroxidase
HPLC	:	High performance liquid chromatography
IPP	:	isoprenyl diphosphate
IPTG	:	isopropyl-beta-D-thiogalactopyranoside
kb	:	kilobase pair
kDA	:	kilodalton

LB	:	Luria-Bertani medium
LCY-b	:	lycopene-β-cyclase
LCY-c	:	lycopene-ε-cyclase
min	:	minute
mRNA	:	messenger RNA
MS	:	Murashige and Skoog medium
NAA	:	naphthaleneacetic acid
NADPH	:	nicotinamide adenine dinucleotide phosphate
NCEDs	:	nine-*cis*-epoxycarotenoid dioxygenases
nm	:	nanometer
nos_{ter}	:	nopaline synthase terminator
NPQ	:	non-photochemical energy dissipation
NXS	:	neoxanthin synthase
OD		optical density (absorbance)
Ospsy	:	rice phytoene synthase cDNA
$Ospsy1_{pro}$	:	rice phytoene synthase 1 promoter
OsPSY	:	rice phytoene synthase (protein)
PAGE	:	polyacrylamide gel electrophoresis
Pchlide	:	chlorophyll tetrapynole precursor protochlorophyllide
PCR	:	polymerase chain reaction
PDS	:	phytoene desaturase
PNK	:	T4 polynucleotide kinase
POR	:	protochlorophyllide oxidoreductase
psy	:	phytoene synthase
PVDF	:	polyvinylidene fluoride
RAE	:	retinol activity equivalent
RAREs	:	retinoic acid response elements
rcf	:	relative centripetal field
RDA	:	recommended daily allowance
RGRC	:	Rice Genome Research Centre
RNA	:	ribonucleic acid
rpm	:	revolution per minute
RT-PCR	:	reverse transcription - PCR
RUBISCO	:	Ribulous 1,5-biphosphate carboxylase oxygenase
SDS	:	sodium dodecyl sulfate
Slpsy	:	tomato phytoene synthase
TAE	:	Tris-acetate/EDTA
TBME	:	*tert*-butyl methyl ether

UNICEF	:	The United Nations Children's Fund
UPLC	:	Ultra pressure (or performance) liquid chromatography
VAD	:	vitamin A deficiency
VDE	:	violaxanthin de-epoxidase
WHO	:	World Health Organization
X-glu	:	5-bromo-4-chloro-3-indolyl-β-glucuronic acid
ZDS	:	ζ-carotene desaturase
ZEP	:	zeaxanthin epoxidase
ZISO	:	ζ-carotene isomerase
Zmpsy	:	maize phytoene synthase
$35S_{pro}$	:	cauliflower mosaic virus 35S promoter
$35S_{pro}m$	:	cauliflower mosaic virus 35S minimal promoter
4-MU	:	4-methylumbelliferone
4-MUG	:	4-methylumbelliferyl-β-D-glucuronide
+	:	positive control
-	:	negative control

Chapter 1: General Introduction

Vitamin A deficiency (VAD) is a global health problem caused by insufficient vitamin A content in diet. According to World Health Organization (WHO), VAD affects more than 118 countries worldwide, and is considered as a serious public health problem in at least 26 countries, especially in highly populated developing countries such as Southeast Asia, Latin America and Africa (Ye *et. al.,* 2000).

Since vitamin A is important and essential for the proper functioning of the visual cycle, immune defense system and epithelial formation, those suffered from VAD are prone to night-blindness and infectious diseases. It is estimated that at least 0.25 million of preschool children go blind every year because of VAD. Besides, children suffered form VAD are more potentially susceptible to fatal afflictions such as diarrhea, respiratory disease and measles (Burkhardt *et. al.*, 1997), contributing to 1 - 3 millions of death every year. Joint efforts from WHO, UNICEF and governments have been carried out to combat VAD by direct supplementation of vitamin A, provision of vitamin A fortified food and food rich in pro-vitamin A carotenoids to those suffered.

Carotenoids are a group of naturally occurring pigments found in plants, photosynthetic algae and bacteria. They contribute to the yellow to red colours of most flowers and fruits. Some of the carotenoids, for instance β-carotene and zeaxanthin, are commonly known pro-vitamin A carotenoids. Once consumed by human, these

carotenoids would be cleaved and converted into vitamin A. It is found that dark-green leaf vegetables and yellow-flesh fruits are rich in pro-vitamin A carotenoids, such as spinach, tomato, mango and papaya. Therefore, supplementation of these vegetables and fruits to those suffered from VAD can alleviate the problem.

Owning to the fact that the staple foods of most of the VAD suffering countries are rice-based, scientists have tried to increase the pro-vitamin A carotenoids content in rice endosperm. Since there are no natural rice varieties with carotenoids-rich endosperm, genetic engineering has to be employed to develop pro-vitamin A enriched rice by filling the gaps of the carotenoids biosynthetic pathway in the grain. This breakthrough is known as the Golden Rice project. The first generation of Golden Rice (GR1) was made by expressing a *Narcissus pseudonarcissusi* phytoene synthase gene (*psy)* and an *Erwinia uredovora* phytoene desaturase gene (CrtI) in the rice endosperm. The GR1 can form yellow grain which accumulates 1.6μg β-carotene per gram of dry endosperm (Ye *et. al.*, 2000). Though the β-carotene content in GR1 was not high enough to fulfill the recommended daily allowance (RDA) of vitamin A by consumption of a reasonable amount of GR1, it demonstrated the feasibility of the principle and brought out the development of the second generation of Golden Rice (GR2).

In GR2, a maize *psy* was used instead of the *Narcissus* copy, which led to a boost in the β-carotene content up to 37μg β-carotene per gram of dry endosperm (Paine *et. al.*,

2005). Due to the simple food matrix of cooked rice, the bioconversion factor of β-carotene of GR2 was high and only 100 grams of uncooked GR2 can provide 500 - 800μg retinol activity equivalent, which was equivalent to 55 - 70% of the RDA for adults (Tang *et. al.*, 2009).

The rice *psy* genes have been identified and characterized recently (Welsch *et. al.*, 2008). Similar to tomato and maize, rice also contains multiple copies of *psy*. Since the PSYs in maize and tomato are known to shown tissue-specific activity: maize PSY1 is specific in endosperm, while the two tomato PSYs are chloroplast- and chromoplast-specific respectively, it is hypothesized that the rice *psy1* and *psy2* may show tissue-specific activity based on their different preferences on membrane association. Besides, as both of the rice *psy1* and *psy2* code for functional enzymes with phytoene-forming activity when tested in bacterial system, they may also be functional in rice endosperm. Thus, it is hypothesized that rice *psy1* and *psy2* can be the potential candidates for making Golden Rice. It is also suggested that the absence of rice *psy* expression in grain is due to insufficient seed-specific elements available in their promoters. Therefore, by introducing seed-specific *cis*-acting element into the rice's *psy* promoter, the seed expression activity may be added.

In this project, to test these hypotheses, we would like to elucidate the tissue-specific activity and the potential of rice *psy1* and *psy2* for making Golden Rice.

On the other hand, we would like to test the feasibility for introducing the seed expression activity by addition of a seed-specific *cis*-acting element, GCN4, into the rice *psy1* promoter.

Chapter 2: Literature Review

2.1 Introduction to carotenoids

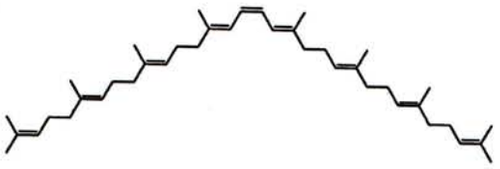
Carotenoids are a group of isoprenoid pigments, and are one of the most wild-spread natural pigments found in all photosynthetic organisms and a few non-photosynthetic bacteria and fungi. In the past few decades, over 600 known carotenoids have been characterized structurally and chemically; however function of most of them is not well defined. For the plant kingdom, carotenoids provide precursor for phytohormone production; also, confer the yellow to red colour to many flowers and fruits, contributing to plant-animal communication (Cunningham and Gantt, 1998). In addition, their anti-oxidizing property makes them play a crucial role in photoprotection (Frank *et. al.,* 1997). Some carotenoids are considered as safe food and cosmetic colourants; and are also used as nutritional supplement in our diet and feed due to their pro-vitamin A property. Recent health-related research has brought the roles of carotenoids in maintaining proper eye function and strengthening immune defense to light. Several studies have revealed the preventative potential of carotenoids against cardiovascular disease and cancer (Fraser *et. al.,* 2004). Owning to the important roles of carotenoids played in plants and animals, the biosynthesis of these isoprenoid pigments has been well elucidated with most of the enzymes involved identified and characterized.

2.1.1 Structures and general chemical properties

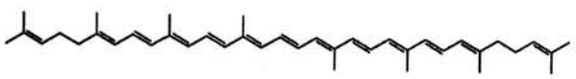
The lipid-soluble carotenoids are characterized by their C_{40} backbone formed by linking eight C_5 isoprene units with or without epoxy, hydroxyl and keto groups. The general chemical structure of the pigment is $C_{40}H_{56}O_n$, where n is the number of oxygen molecule ranging from 0 - 6. Carotenoids, as shown in Figure 2.1, without oxygen molecule are known as carotene, such as phytoene, lycopene, α- and β-carotene; while those having oxygen molecule are known as xanthophylls including lutein, zeaxanthin, violaxanthin and neoxanthin. Cyclization of the carbon skeleton forms a ring structure at one or both ends, giving carotenoids such as α- and β-carotene.

The extensive central double bond structure in the polyene chain, usually from 3 - 15, contributes to light absorption. The length of this structure determines the colour of carotenoids to eye. Furthermore, this extensive double bond structure and some of the end groups may be susceptible to free radicals and reactive oxygen species attack, giving the anti-oxidant property to some carotenoids (El-Agamey *et. al.,* 2004). Finally, some carotenoids sharing the α- and β-ring structures, see Figure 2.1, are also known to be the precursor of vitamin A. These pro-vitamin A species can be converted into vitamin A by an oxygenase in our digestive system (Fraser *et. al.,* 2004).

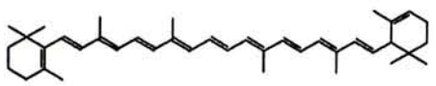
Carotenes:



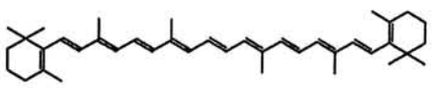
Phytoene



Lycopene



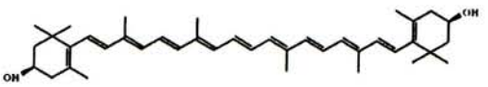
α-carotene



β-carotene

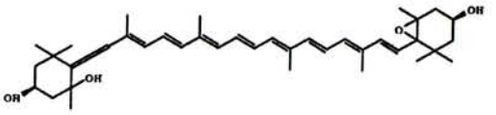
Xanthophylls:

Lutein



Zeaxanthin

Violaxanthin



Neoxanthin

Figure 2.1 Chemical structures of carotenoids

Carotenoids without oxygen are grouped as carotenes; while those having oxygen are grouped as xanthophylls

2.1.2 Dietary sources of carotenoids

Since all animals and human lack the biosynthetic pathway, we cannot make carotenoids *de novo* and have to depend on dietary carotenoids. Crop plants are the primary carotenoid source in human diet, where different carotenoids are stored in the roots, leaves, seeds and fruits. For instance, carrot is rich in α- and β-carotene, the dark-green leafy spinach is rich in lutein, corn grain is rich in zeaxanthin, and tomato, watermelon and papaya are rich in lycopene. It is found that around 60 carotenoids species have been identified in fruits and vegetables normally consumed by humans. The fat-soluble carotenoids can also be obtained from animal sources, such as lutein in the egg yolk (Scott and Hart, 1994).

2.2 Biosynthesis of carotenoids in plants

The biosynthetic pathway of carotenoids in plants (see Figure 2.2) has been extensively elucidated since 1950s. Thanks for the recent advances in molecular genetics, the use of molecular tools helped discovering nearly all the genes encoding the enzymes involved in the pathway, and the activities and co-factor requirement of most of them have been well characterized.

The carotenoid biosynthesis takes place in the plastids such as the chloroplast in leaves, chromoplast in coloured flowers and fruits and amyloplast in seed endosperm. In leaf, the biosynthesis of carotenoids is primarily associated with light-harvesting and

photoprotection of the photosystem complexes (Fraser *et. al.,* 2004). In ripening-fruit and flower, the production of carotenoids is associated with the aroma and colour of these organs; while in the root, carotenogenesis is proposed to be related with stress-tolerance (Li *et. al.,* 2008a and Welsch *et. al.,* 2008).

Existing model of carotenogenesis is suggested to be happened on the plastid membrane within multienzyme complexes (Al-Babili *et. al.,* 1996 and Cunningham and Gantt, 1998) depicted in Figure 2.3. This multienzyme complexes model provides a hydrophobic region which can assist the lipid-soluble substrates channelling from one enzyme to another; and hence, improving the efficiency of production (Beyer *et. al.,* 1985 and Bonk *et. al.,* 1997). It is also suggested that there may be other peptides and ions associated with this complexes as cofactors.

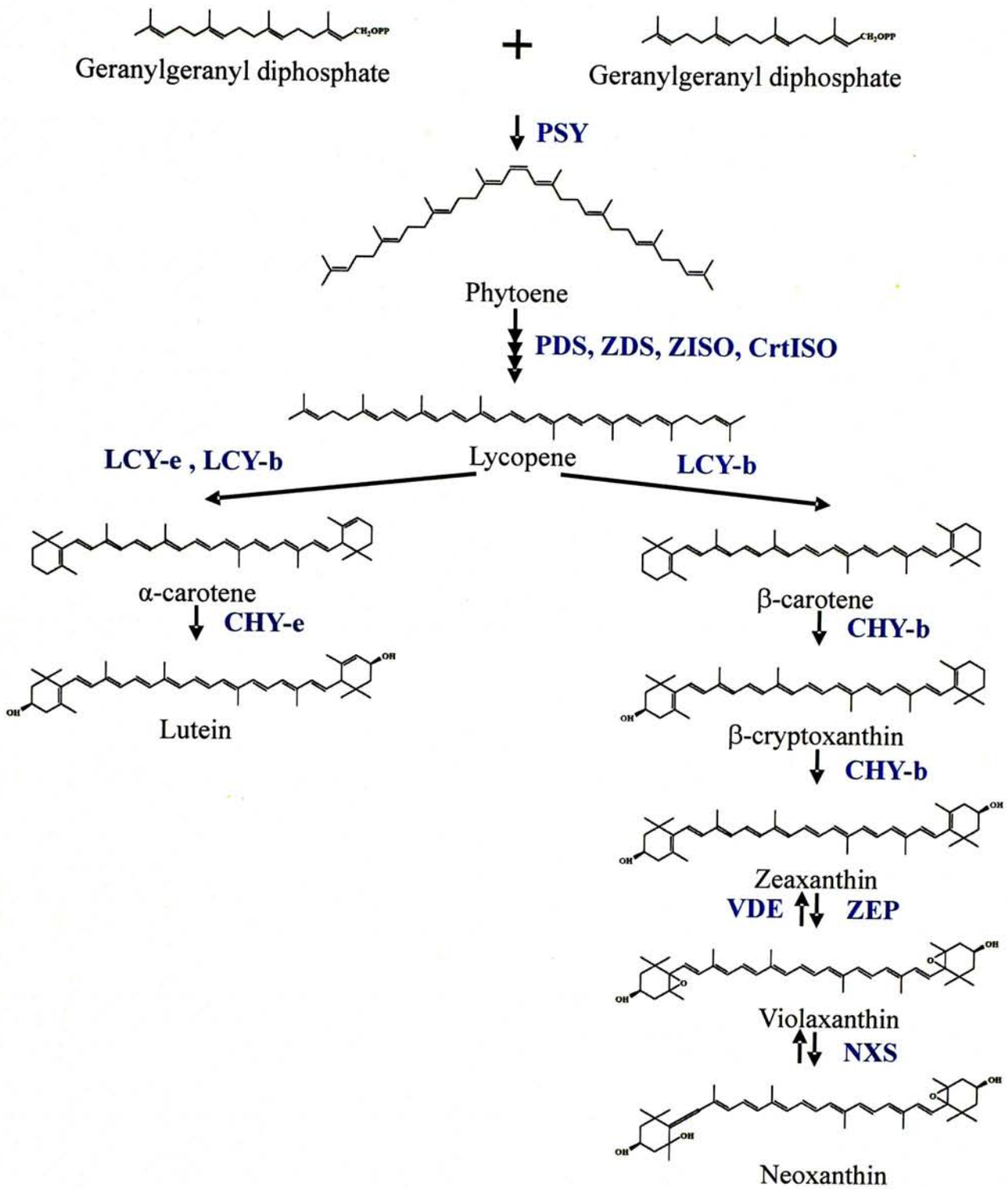


Figure 2.2 The biosynthetic pathway of carotenoid in higher plant

Abbreviations: carotenogenic enzymes (blue)

PSY	:	phytoene synthase	LCY-b	:	lycopene-β-cyclase
PDS	:	phytoene desaturase	CHY-e	:	ε-hydroxylases
ZDS	:	ζ-carotene desaturase	CHY-b	:	β-ring hydroxylases
ZISO	:	ζ-carotene isomerase	ZEP	:	zeaxanthin eopxidase
CrtISO	:	carotene isomerase	VDE	:	violaxanthin de-epoxidase
LCY-e	:	lycopene-ε-cyclase	NXS	:	neoxanthin synthase

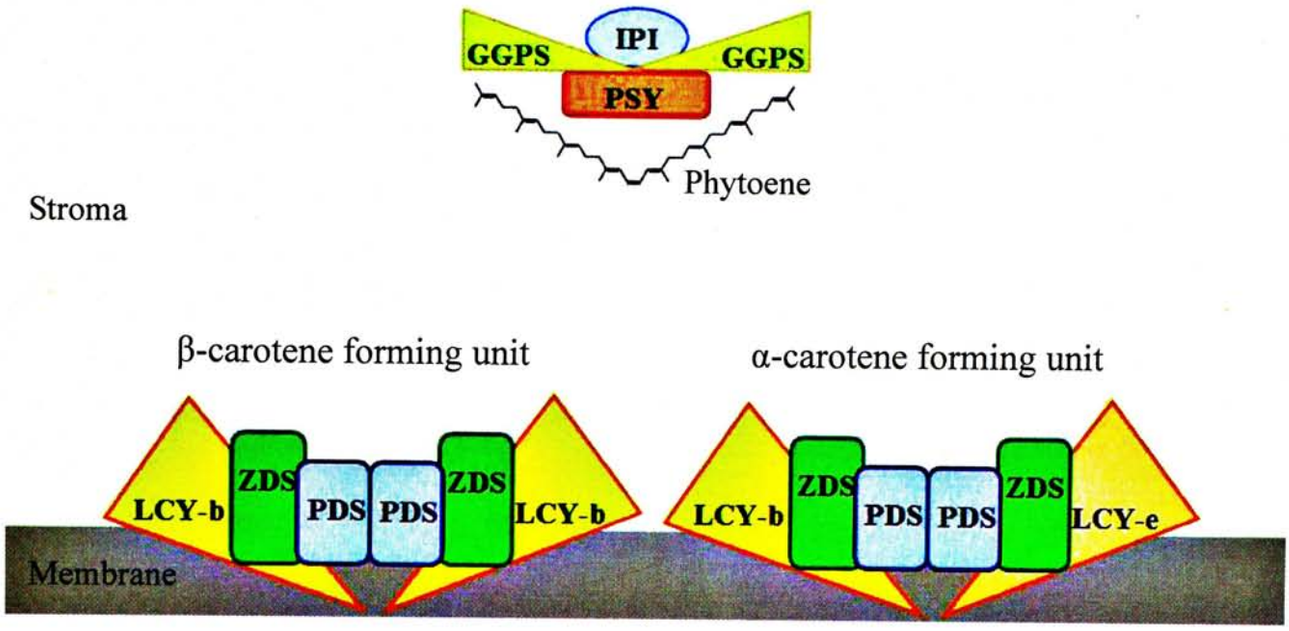


Figure 2.3 Schematic diagram of hypothetical carotenogenic multienzyme complexes in plastid membranes and stroma (re-drawn from Cunningham and Gantt, 1998)

Abbreviations:

IPI	:	IPP isomerase	GGPS	:	geranylgeranyl diphosphate synthase
PSY	:	phytoene synthase	PDS	:	phytoene desaturase
ZDS	:	ζ-carotene desaturase	LCY-b	:	lycopene-β-cyclase
LCY-e	:	lycopene-ε-cyclase			

2.2.1 Formation of isopretenyl diphosphate (IPP)

In higher plants, the synthesis of isoprenoids such as carotenoids, gibberellins, tocopherols, chlorophylls and phylloquinones share the common five-carbon, C_5 building block, isoprenyl diphosphate (IPP). In the cytoplasm, IPP is formed by the mevalonate-dependent pathway from the plastid-originated acetyl-CoA. The formation of plastid isoprenoids at some developmental stages arise partly from this pathway (Kasahara *et. al.*, 2002).

The formation of IPP for the plastid-derived isoprenoids is suggested to be provided mainly from the mevalonate-independent pathway, see Figure 2.4. In this pathway, 1-deoxy-D-xylulose 5-phosphate (DXP) is formed by a head to head condensation of pyruvate derived thiamine and D-glyceraldehyde-3-phosphate (G3P) by the 1-deoxy-D-xylulose 5-phosphate synthase (DXPS). Then an intramolecular rearrangement and reduction of DXP forms 2-C-methyl-D-erythritol 4-phosphate (MEP) by DXP reductioisomerase (DXR) in the presence of NADPH and Mn^{2+}. After that, MEP is converted into 1-hydroxy-2methyl-2-(E)-butenyl 4-phosphate (HMBPP) via a series of reactions from 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-ME), 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-MEP), and 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEP-cPP) catalyzed by 2-C-methyl-D-erythritol 4-phosphate cytidyl transferase (MCT), 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase (MCK), 2-C-methyl-D-erythritol

2,4-cyclodiphosphate synthase (MCS) and 1-hydroxy-2-methyl-2-2(E)-butenyl 4-phosphate synthase (HDS) . Finally, under the action of IPP/DMAPP synthase (IDS), a 5:1 mixture of isoprenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are formed by HMBPP.

2.2.2 Formation of C_{40} backbone, phytoene

A C_{20} geranylgeranyl diphosphate (GGPP) is the precursor for carotenoids production. This C_{20} compound is formed by a serious of condensation of four C_5 isoprene units as depicted in Figure 2.5. The chain elongation reaction starts by the isomerization of IPP into its allylic isomer, DMAPP by IPP isomerase (IPI). Then, a condensation of DMAPP and IPP under the action of geranyl diphosphate synthase (GPS) results a C_{10} geranyl diphosphate (GPP). Further condensation of two IPP sequentially by geranylgeranyl diphosphate synthase (GGPS) forms the C_{15} farnesyl diphosphate (FPP), and finally, the C_{20} GGPP. Both IPI and GGPS require the presence of Mn^{2+} ion for proper function (Fraser *et. al.,* 2000; Maudinas *et. al.,* 1975; Dogbo and Camara, 1987 and Lutzow *et. al.,* 1988). The first committed step of carotenogenesis is the condensation of two GGPP by the action of phytoene synthase (PSY) in the presence of Mn^{2+} ion, and for PSY in some plants require ATP as well (Than *et. al.,* 1972; Dogbo *et. al.,* 1988 and Schledz *et. al.,* 1996), forming phytoene, the C_{40} backbone of all carotenoids, as shown in Figure 2.2.

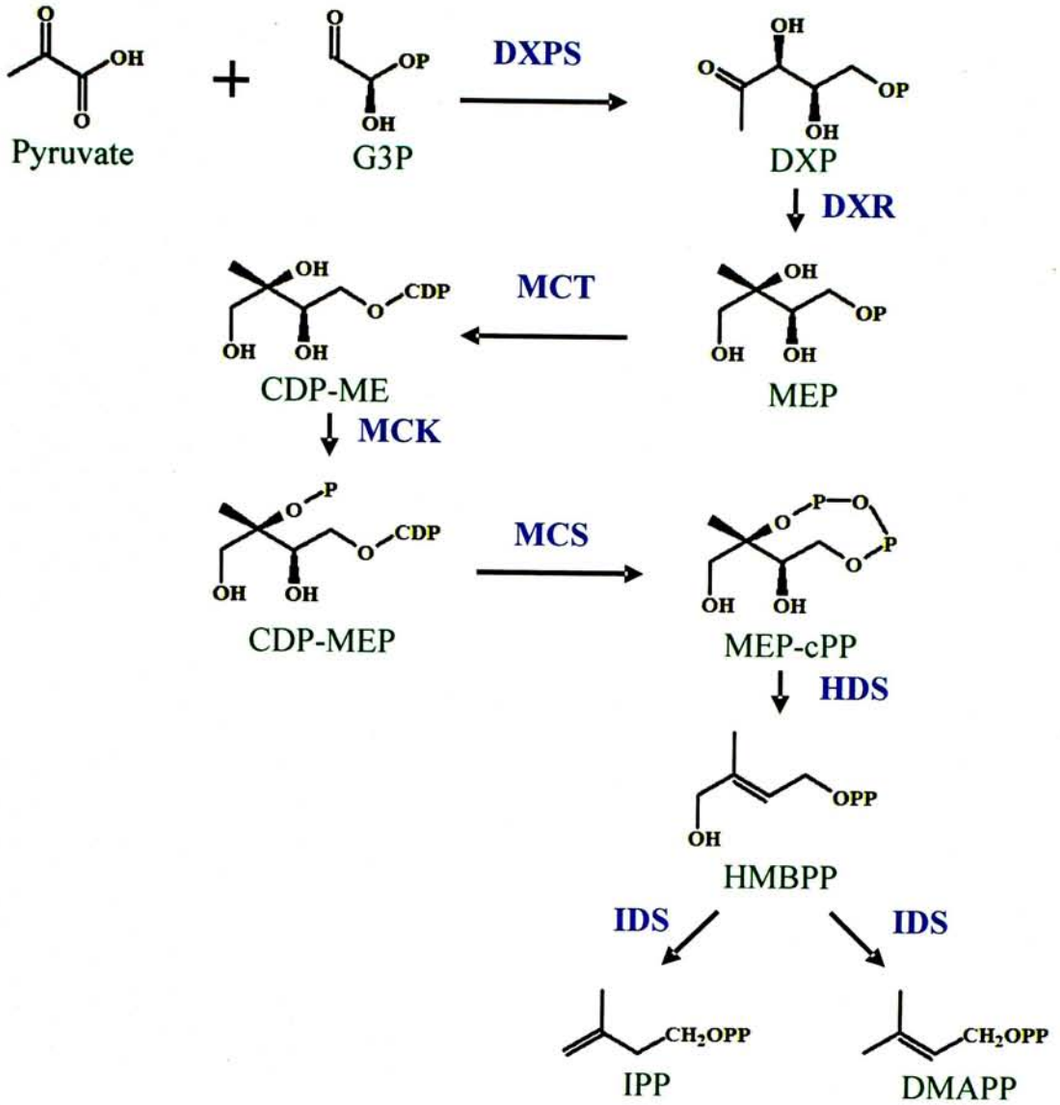


Figure 2.4 Pathway of the formation of isoprenyl diphosphate (IPP)

Abbreviations: enzymes (blue), intermediate (green)

DXPS	:	1-deoxy-D-xylulose 5-phosphate synthase
DXR	:	DXP reductioisomerase
MCT	:	2-C-methyl-D-erythritol 4-phosphate cytidyl transferase
MCK	:	4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase
MCS	:	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
HDS	:	1-hydroxy-2-methyl-2-2(E)-butenyl 4-phosphate synthase
IDS	:	IPP/DMAPP synthase
G3P	:	D-glyceraldehyde-3-phosphate
DXP	:	1-deoxy-D-xylulose 5-phosphate
MEP	:	2-C-methyl-D-erythritol 4-phosphate
CDP-ME	:	4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol
CDP-MEP	:	2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol
MEP-cPP	:	2-C-methyl-D-erythritol-2,4-cyclodiphosphate
HMBPP	:	1-hydroxy-2methyl-2-(E)-butenyl 4-phosphate
IPP	:	isoprenyl diphosphate
DMAPP	:	dimethylallyl diphosphate

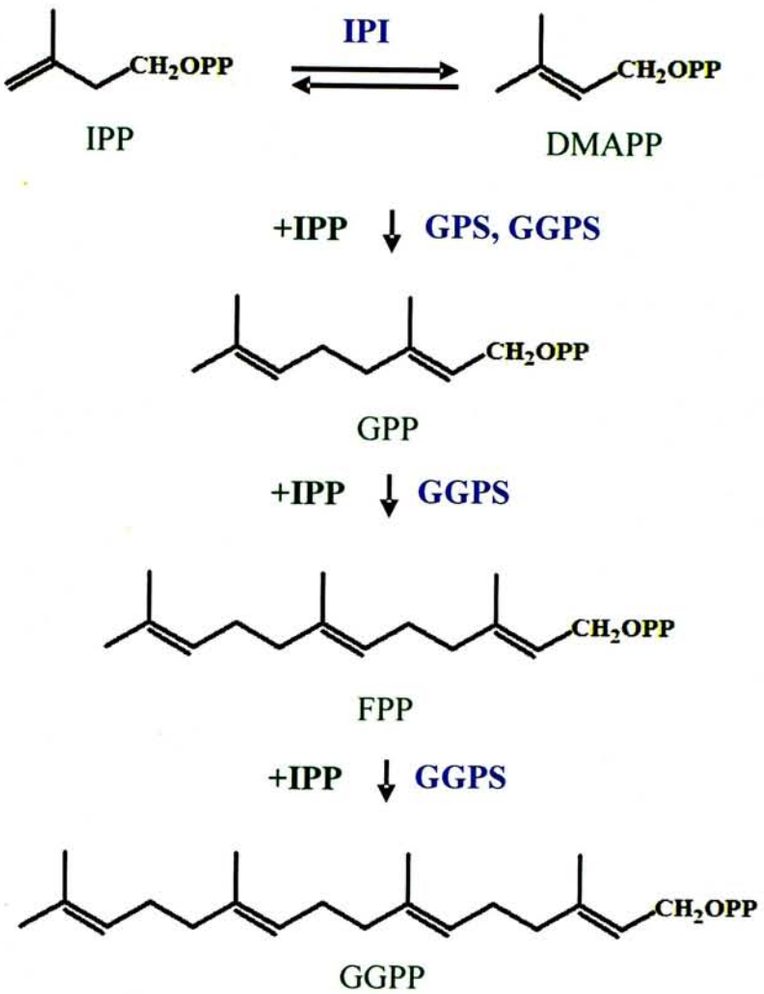


Figure 2.5 Formation of C_{20} geranylgeranyl diphosphate (GGPP) from IPP and DMAPP

Abbreviations: enzymes (blue), intermediate (green)

IPI	:	IPP isomerase
GPS	:	geranyl diphosphate synthase
GGPS	:	geranylgeranyl diphosphate synthase
IPP	:	isoprenyl diphosphate
DMAPP	:	dimethylallyl diphosphate
GPP	:	geranyl diphosphate
FPP	:	farnesyl diphosphate
GGPP	:	geranylgeranyl diphosphate

2.2.3 Desaturation reactions

After the formation of phytoene series of desaturation reactions take place to introduce a series of four conjugated carbon-carbon double bonds, responsible for the distinct chromophore of carotenoid pigments. These four desaturation steps form phytofluene, ζ-carotene, neurosporene and lycopene sequentially catalyzed by two enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), in the presence of FAD and quinines (Fraser *et. al.,* 2000; Camara *et. al.,* 1982; Beyer *et. al.,* 1985 and Breitenbach *et. al.,* 1999), as depicted in Figure 2.6. The extended chromophore converts colourless phytoene into red lycopene.

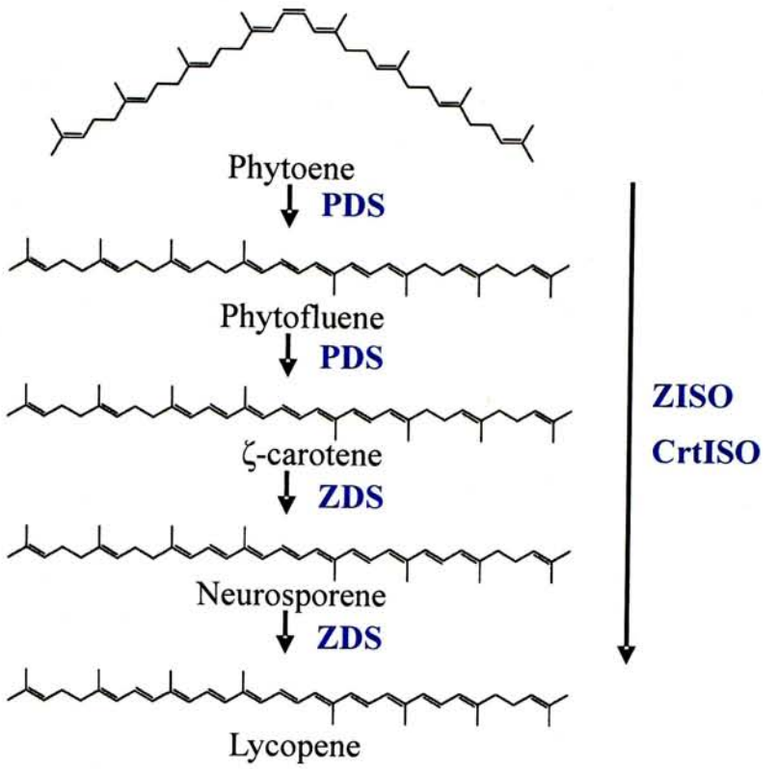


Figure 2.6 Desaturations reaction of phytoene

Abbreviations: carotenogenic enzymes (blue)

PDS	:	phytoene desaturase	ZDS	:	ζ-carotene desaturase
ZISO	:	ζ-carotene isomerase	CrtISO	:	carotene isomerase

2.2.4 Isomerization

In the plant system, since phytoene exists in the 15-*cis*-isomer predominantly, the product of PDS and ZDS is the poly-*cis*-lycopene, also known as prolycopene. Due to the light-induced isomerization, and the action of carotene isomerase (CrtISO) and ζ-carotene isomerase (ZISO), all poly-*cis*-lycopene are converted into all-*trans*-lycopene (Park *et. al.,* 2002 and Li *et. al.,* 2007).

2.2.5 Cyclization

Lycopene is the substrate of two competing cyclization reactions catalyzed by the lycopene-β-cyclase (LCY-b) and lycopene-ε-cyclase (LCY-e) in the presence of NADPH, in which a six-membered ring is formed at one or both ends of the acyclic carotenoid. The cyclization of both ends by LCY-b introduces two β-rings giving the β-carotene; while, the cyclization of both ends by LCY-b and LCY-e respectively introduce a β-ring and a ε-ring, giving the α-carotene (Fraser *et. al.,* 2000; Beyer *et. al.,* 1991 and Camara and Dogbo, 1986). This cyclization step gives rise to the first brunching point in carotenoid biosynthesis as shown in Figure 2.2.

2.2.6 Xanthophylls synthesis

2.2.6.1 Hydroxylation

Xanphothylls, also known as oxygenated carotenoids, are predominantly found in the thylakoid membranes serving for different functions. The hydroxylation of C-3 carbon of each ring of β-carotene and α-carotene forms zeaxanthin and lutein respectively, as shown in Figure 2.2. This hydroxylation is catalyzed by hydroxylases specific for the β- and ε-rings in the presence of ferredoxin and iron. To date, two nonheme diiron β-ring hydroxylases (CHY-b) have been identified from *Arobidopsis* for the hydroxylation specifically in the β-ring (Lange and Ghassemian, 2003 and Sun *et. al.*, 1996); while, hydroxylation of the ε-ring is proposed to be carried out by two P450-type ε-hydroxylases (CHY-e) (Kim and DellaPenna, 2006).

2.2.6.2 Epoxidation and de-epoxidation

Violaxanthin is formed from zeaxanthin via antheraxanthin by zeaxanthin eopxidase (ZEP) in the presence of reduced ferredoxin. In this conversion, 5,6-epoxy groups are introduced into the 3-hydroxy-β-ring. Such conversion of zeaxanthin to violaxanthin is reversible and is catalyzed by violaxanthin de-epoxidase (VDE) in the presence of ascorbate, as shown in Figure 2.2. These reversible reactions are collectively known as the violaxanthin cycle or the xanthophylls cycle (Yamamoto, 1985), which plays an important role in photoprotection in the photosynthetic organ.

2.2.6.3 Neoxanthin formation

Neoxanthin is formed from violaxanthin by neoxanthin synthase (NXS). The NXS of potato (Al Babili *et. al.,* 2000) and tomato (Bouvier *et. al.,* 2000) have been identified and characterized. However, no NXS have been identified from the *Arobidopsis* yet.

2.2.7 Carotenoids catabolism by cleavage enzymes

A cleavage dioxygenase family has been identified in different plant's genome, such as *Arabidopsis* and rice. These cleavage dioxygenases can be classified in two groups: the nine-*cis*-epoxycarotenoid dioxygenases (NCEDs) and carotenoid-cleaving dioxygenases (CCDs) (Welsch *et. al.,* 2008). The NCEDs are responsible for cleaving the 9-*cis*-isoform of violaxanthin and neoxanthin for the biosynthesis of phytohormone, abscisic acid (ABA) (Zeevaart *et. al.,* 1988). On the other hand, CCDs exhibit various regional cleavage specificities and end group preferences. Some of them are involved in the production of root-derived long-range signalling molecules on lateral branching (Welsch *et. al.,* 2008).

2.2.8 Carotenoids sequestration

After carotenogenesis, carotenoids produced are deposited into different organelles in various forms depends on the type of plastid where they are made. In the chloroplasts, the end-products carotenoids, for example, lutein, zeaxanthin and violaxanthin, are associated with the light-harvesting complexes. They are deposited on the thylakoid

membrane for photo-harvesting and photoprotection; while, the unbound carotenoids are associated with specific protein forming the plastoglobules (Kuhlbrandt, 1994).

As for those produced in the chromoplast, different plants show different sequestration of carotenoids. In the fruit of pepper, the end-product carotenoids are esterified and associate with a fibrillin protein as fibrils (Deruere *et. al.,* 1994); while, lycopene in tomato is sequestrated in crystal form (Shewmaker *et. al.,* 1999). These ways of carotenoid sequestration help sorting the end-product carotenoids away from where they are formed; and hence, preventing end-product inhibition. Also, these measures can help protecting the carotenoids from cleaving by CCDs, NCEDs and degradation by light and oxygen in the environment.

2.2.9 Regulations of Carotenogenesis in plant

Recent studies have also focused on the regulations of carotenogenesis; and it is proposed that there are at least three ways to control the biosynthesis of carotenoids in different tissues.

The first mechanism is the control of expression of genes for carotenogenesis. Although no transcriptional factors responsible for the expression of carotenoids biosynthetic genes have been identified, experiments have been carried out to show the light-induced expression of PSY in mustard (Welsch *et. al.,* 2000) and rice (Welsch *et.*

al., 2008). In both cases, there are a phytochrome-regulated up-regulation of PSY mRNA and protein after illumination by white and far-red radiations in mustard, and by white and red radiations in rice. Light-induced *cis*-acting elements, such as Box I element (TTTCAAA), Box IV element (TAATTAAT) and AE Box (AGAAACAA) have been identified in the promoters of rice PSY. Besides, overexpression of a blue light photoreceptor CRY2 can result in an increase of carotenoid and flavonoid levels in tomato (Giliberto *et. al.,* 2005); similarly, silencing of the negative DDB1, DET1 and COP1-like regulators increases lycopene levels in tomato (Liu *et. al.,* 2004 and Davuluri *et. al.,* 2004).

On the other hand, stress-induced root-specific expression of PSY, ZEP and NCED1 of maize (Li *et. al.,* 2008a), and PSY, ZEP and NCED4 in rice (Welsch *et. al.,* 2008) have been demonstrated. The identification of ABRE (ABA-response element) in the promoter of a rice PSY suggested the expression of this gene under abiotic stresses, for instance, salt, drought and low temperature.

It is interesting that more than one carotenogenesis enzyme are encoded by multiple copies of gene in different plants, for instance, two GGPSs in tomato, two PSYs in tomato (Galpaz *et. al.,* 2006), three PSYs in maize (Li *et. al.,* 2008a) and rice (Welsch *et. al.,* 2008), two LCY in tomato (Hirschberg *et. al.,* 2001), and, two and three β-hydroxylases in *Arabidopsis* (Rissler and Pogson, 2001) and tomato (Liu *et. al.,* 2003)

respectively. In tomato, only one isoform of GGPS, PSY, LCY and β-hydroxylase are constitutively expressed in and specific for chromoplast in flower and fruit. These existences of multigene families allow the establishment of a tissue-specific carotenogenesis pathway: a carotenogenesis pathway in chromoplast that is parallel to the one in chloroplast. Such phenomenon allows a controlling mechanism of carotenoids production in flower and fruit without affecting the carotenoids pattern in other parts of the plant, such as those in photosynthetic organ, where the biosynthesis of carotenoids is strictly regulated (Taylor *et. al.,* 2005).

Finally, there are different ways, classified as post-transcriptional regulation, to control the activation and activities of the carotenogenesis enzymes; for example, the requirement of Mn^{2+} ion and specific membrane association of PSY activity (Schledz *et. al.,* 1996), the light-induced membrane association of PSY during photomorphogenesis (Park *et. al.,* 2002), and the substrate specificity of LCY for the partitioning of β- or ε-ring carotenoids. Also, a direct inhibition of enzyme activity by carotenoid end products is suggested as the major form of regulation in flower and fruit (Fraser and Bramley, 2004).

2.3 Roles of carotenoids in plants

As described above, carotenoids are a group of isoprenoid pigments with distinct chemical and biological properties; they are involved in a series of physiological activities of plants.

For example, the attractive colour of fruits and flowers are contributed by the accumulation of yellow to red carotenoids mixture in the chromoplast. Also, some carotenoids act as a precursor for producing ionones, damascones and damascenones, such as β-ionone and β-damascenone which give the fragrance of rose, by carotenoid degradation. By adding distinctive colour and scent to flowers and fruits, carotenoids assist plant-animal communication; and hence, pollen and seed dispersal (Howitt and Pogson, 2006).

Besides fragrance, carotenoids are involved in other plant physiology, for instance, the production of seed dormancy and germination controlling abscisic acid, the structure stabilization of etioplasts for chloroplast formation, and the light-harvesting and photoprotection of photosystem in photosynthetic tissues.

2.3.1 Precursor of abscisic acid (ABA) production

Carotenoids derivatives, 9-*cis*-violaxanthin, 9-*cis*-neoxanthin and 9-*cis*-zeaxanthin are the precursor for biosynthesis of phytohormone abscisic acid where NCEDs are involved (Zeevaart *et. al.,* 1988). These 9-*cis* isoforms are cleaved by NCED forming

xanthoxin, which is then converted into ABA via two oxidation steps subsequently, as depicted in Figure 2.7.

It has been demonstrated that in the root of maize and rice, upon drought and salt treatments mimicking abiotic stress situations, the transcript levels of carotenogenic enzymes such as PSY, ZEP and β-ring hydroxylases increased; and hence the carotenoid level, proceeding ABA production. It is suggested that the stress-induced production of carotenoids helps replenishing the xanthophylls pool in root for ABA biosynthesis (Li *et. al.*, 2008a and Welsch *et. al.*, 2008).

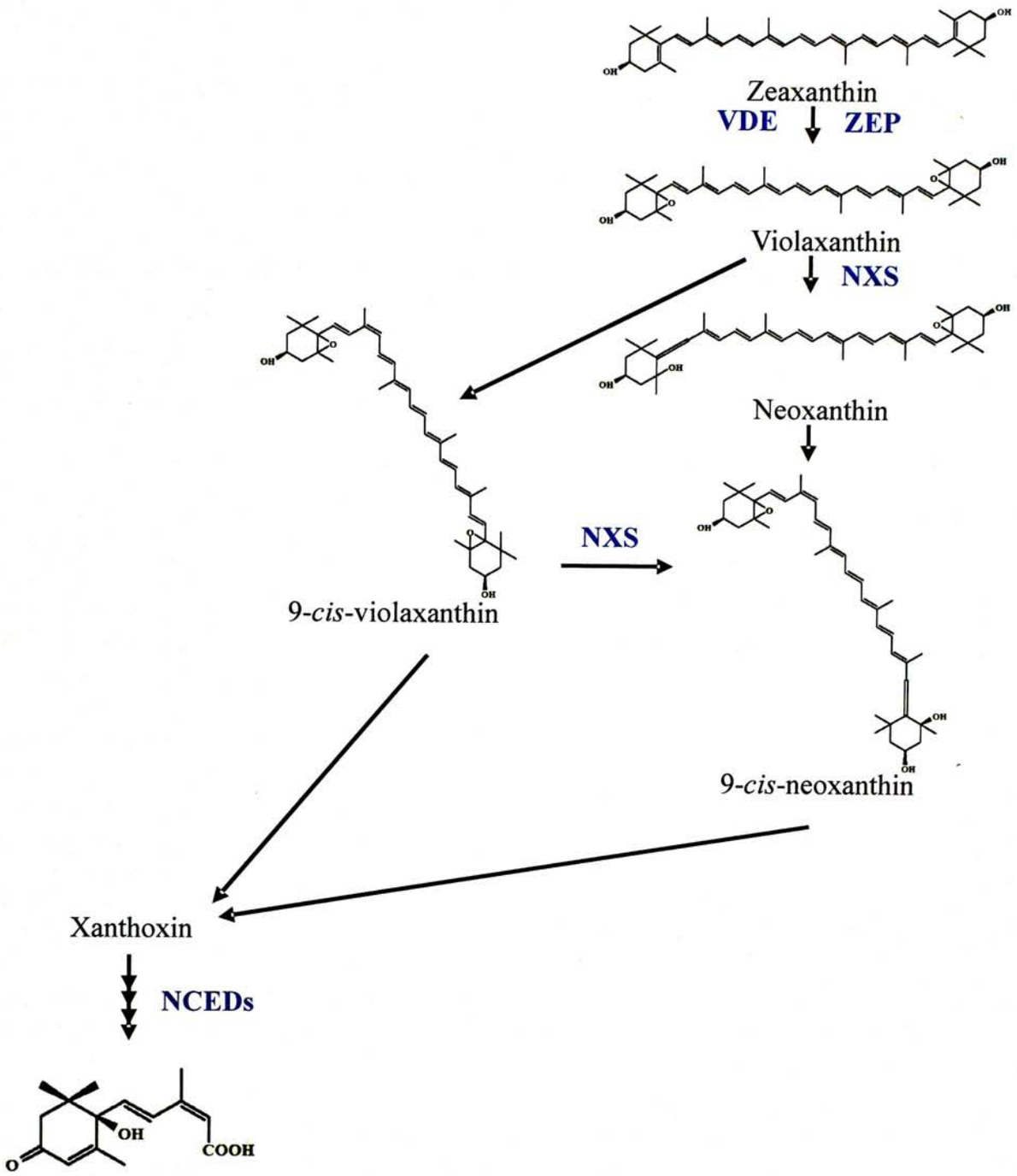


Figure 2.7 The biosynthesis of abscisic acid

Abbreviations: carotenogenic enzymes (blue)

ZEP	:	zeaxanthin eopxidase	VDE	:	violaxanthin de-epoxidase
NXS	:	neoxanthin synthase	NCEDs	:	nine-*cis*-epoxycarotenoid dioxygenases

2.3.2 Photomorphogenesis: Prolamella body formation

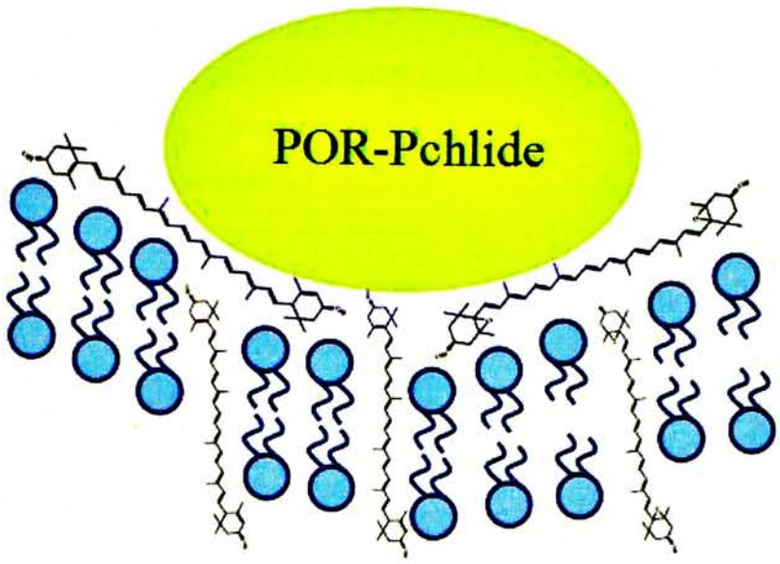
In dark-grown plants or in germinating seedling, there is an important organelle known as etioplast. Etioplast is a plastid containing necessary components and building blocks for making chloroplast. Inside the etioplast, there is a uniformly curved lattice tubular membrane structure, prolamellar body, which is associated with protein complexes, POR (protochlorophyllide oxidoreductase) : Pchlide (chlorophyll tetrapynole precursor protochlorophyllide). Upon illumination, the prolamellar body would decay and disperse; then, the formation of thylakoid, photosystem assembly, carotenoid and chlorophyll biosynthesis follow, giving the functional chloroplast. It is suggested that the etioplasts and prolamellar body facilitate a rapid chlorophyll formation upon illumination; thus, giving photosynthetic competence to the seedling once it emerges from the soil into the light.

Carotenoids play crucial roles in this photomorphogenesis event. Experiment has been conducted to show that upon illumination, an up-regulation of PSY gene and a migration of PSY enzyme to the newly formed thylakoid were detected, adding a functional, membrane-associated carotenoid biosynthesis pathway to the thylakoid. The production of PSY enzyme was followed by chlorophyll formation and photosynthetic apparatus assembly, suggesting that PSY activity guided the time to start etioplast transformation into chloroplast (Welsch *et. al.*, 2000).

In addition, lutein and violaxanthin are found to be important structural

components for the prolamellar body. By studying a mutant mustard seedling accumulates poly-*cis*-carotenoids instead of lutein and violaxanthin, the presence of stepped carotenoids destabilized the curved structure and subsequently delayed the formation of chloroplast after illumination, as depicted in Figure 2.8b. Therefore, it is suggested that lutein and violaxanthin are necessary for regulating the membrane fluidity of prolamellar body for stabilizing the curved structure once interact with the POR:Pchlide complexes (Park *et. al.*, 2002), as depicted in Figure 2.8a.

(A) In wild-type mustard seedling



(B) In mutant mustard seedling

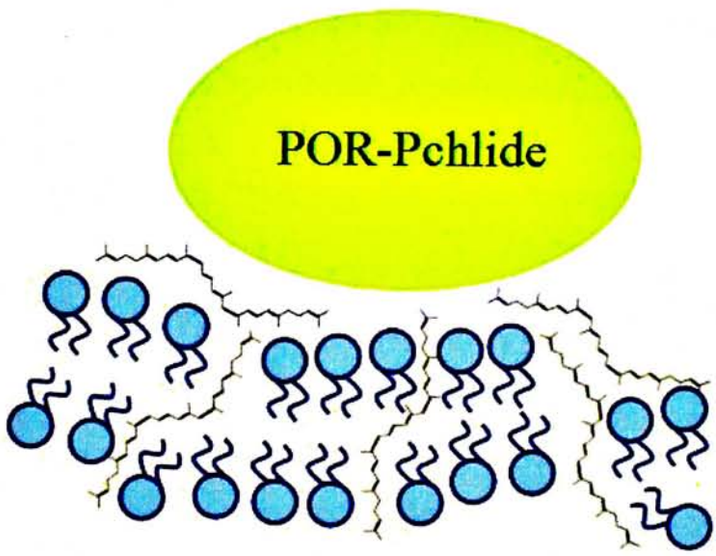


Figure 2.8 Schematic diagram of hypothetical interaction between membrane, carotenoids and POR:Pchlide in prolamellar body formation (re-drawn from Park *et. al.*, 2002)

(A) In wild-type mustard seedling

(B) In mutant mustard seedling

2.3.3 Light-harvesting, energy transfer and photoprotection

One of the most important and distinctive physiological mechanisms of the plant kingdom is photosynthesis by which plants can make use of the solar energy, water and atmospheric carbon dioxide to produce their own energy source and storage in a form of starch. In order to undergo a successful photosynthesis, plants have to evolve and develop a set of measures that can efficiently collect light and transfer the energy to the reaction centre; and on the other hand, prevent photoinhibition and photooxidation by excited chlorophyll and reactive oxygen species generated during oxygenic photosynthesis. Different carotenoids play a role in these measures.

Photosynthesis takes place in the photosystems complexes located on the thylakoid membrane of chloroplast. Integral membrane proteins and carotenoids act as structural and light-harvesting components, holding the light-harvesting complex within the thylakoid membrane, so that the associated chlorophyll molecules can absorb solar energy. Lutein has been identified to be one of the most abundant carotenoids in the photosystem. As lutein has a roughly isoenergetic excited energy state with that of chlorophyll a (Frank *et. al.,* 1997), it is suggested that lutein can act as an accessory pigment to absorb light and transfer energy efficiently to chlorophyll a within a photosynthetic antenna system.

During the course of photosynthesis, photosystem is excited by solar energy. When the incident energy is higher than that required, excess energy may produce reactive

intermediates such as triplet state chlorophyll, singlet oxygen and reactive oxygen species. These reactive intermediates may cause oxidative damage to the photosynthetic apparatus and reduce productivity, resulting as photoinhibition and photooxidation. Therefore, plants have developed a series of repair and protective systems. Long term protective systems include the adjustment of light-harvesting antenna size, the accumulations of lipophilic or water-soluble anti-oxidant molecules, and the accumulation of sunscreen molecules such as anthocyanins (Havaux and Kloppstech, 2001). In contrast, carotenoids take part in a relative rapid and immediate short term system in which the excessive energy is safely dissipated as heat and is known as non-photochemical energy dissipation (NPQ) as depicted in Figure 2.9.

NPQ is a short-term adaptation to changes in light density, which is obligatorily dependent on the presence of zeaxanthin and antheraxanthin. Upon high illumination, excess excited singlet chlorophylls are formed. At the same time, the pH within the photosynthetic membrane is lowered by an influx of proton. Protonation shows dual functions: first, it induces the biochemical conversion of violaxanthin to zeaxanthin by de-epoxidation of the xanthophylls cycle within the photosystem; second, binding of protons to the photosystem acts synergetically with the photo-transformation of violaxanthin to zeaxanthin which induces a configuration change in the structure of the photosystem that is necessary for thermal dissipation of excess energy (Demmig-Adams

and Adams, 1996). These actions prevent excess energy from reaching the photosystem reaction centre and deactivate excited chlorophyll or quench chlorophyll fluorescence. On the other hand, the zeaxanthin formed is released to the lipid phase of thylakoid membrane in which zeaxanthin acts together with vitamin E presence to scavenge reactive oxidative species.

There are two proposed models of fluorescence quenching. The first one is the molecular gear shift model involving a direct carotenoids and excited chlorophyll interaction. As the energy state of excited chlorophyll a is higher than that of zeaxanthin, it is suggested that a thermo-dynamically feasible and direct downhill energy transfer from excited singlet chlorophyll a to zeaxanthin, which is finally dissipated as heat. The second one is the light-harvesting complex model which proposes indirect energy dissipation via zeaxanthin. In this model, both association of violaxanthin or zeaxanthin could lead to dissipation of excess energy but at different extents and with different level of pH changes. It is suggested that zeaxanthin can amplify fluorescence quenching in the light-harvesting complex in which a lower pH change across the membrane is required and more energy could be dissipated as heat when compared with violaxanthin (Young *et. al.,* 1997).

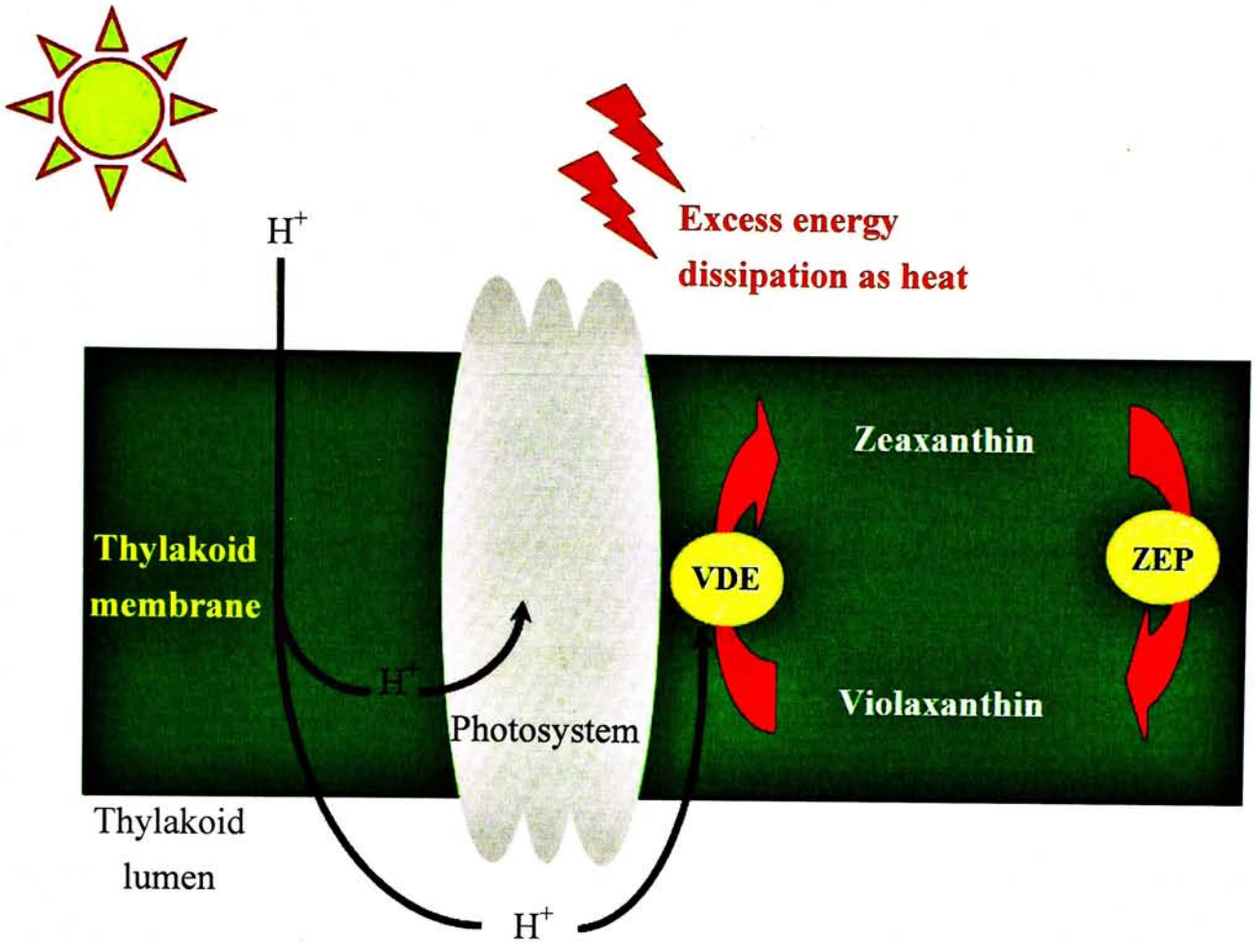


Figure 2.9 Schematic diagram of non-photochemical energy dissipation and biochemistry of the xanthophylls cycle induced by pH change upon illumination
(re-drawn from Demmig-Adams and Adams, 1996)

Abbreviations:

ZEP : zeaxanthin eopxidase VDE : violaxanthin de-epoxidase

2.4 Importance of carotenoids to human

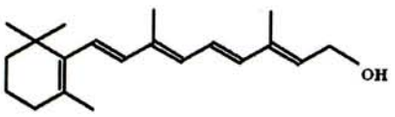
2.4.1 Provitamin A activity and conversion

Vitamin A is one of the micronutrients that is involved in different metabolisms, hence is important and necessary for maintenance of health of our body. Vitamin A is a group of compounds that exhibit biological activities similar to that of all-*trans* retinol, including retinal, retinyl esters and retinoic acids (Blomhoof and Blomhoff, 2006) (see Figure 2.10). Since our body cannot synthesize vitamin A and its derivatives *de novo*, we have to obtain it from diet. Animal products such as egg yolk, milk, cheese and liver are rich in vitamin A.

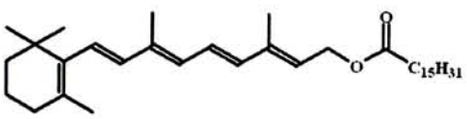
Some of the plant derived carotenoids are known to possess pro-vitamin A activity. Although there are 600 known carotenoids, about 50 with the β-ring end show pro-vitamin A activity and only a few of them could be used as precursor of vitamin A in our body. Carotenoids such as β-carotene, β-cryptoxanthin and zeaxanthin (see Figure 2.10), can be cleaved by an intestinal 15-15'-diosygenase, reported in human, chicken and *Drosophila*, to form vitamin A in our digestive system (Kiefer *et. al.,* 2002; Wyss *et al.*, 2000 and von Lintig and Vogt, 2000). β-carotene is the most ubiquitous carotenoid and is the most efficient in its bioconversion into vitamin A by which a single central cleavage yields two retinal molecules (Nagao, 2004). In addition to liver and digestive system, the human dioxygenase enzyme is also found to be expressed in non-digestive

tissues where carotenoids delivered by the plasma are converted into retinal locally (Lindqvist and Andersson, 2002).

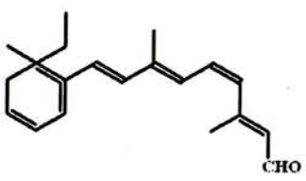
Vitamin A – Active Retinoids:



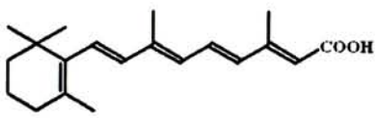
all - *trans* retinol



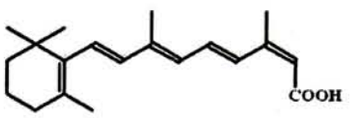
Retinyl palmitate



11 - *cis* - retinal

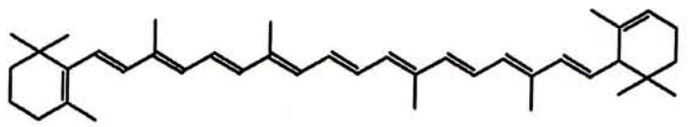


All - *trans* retinoic acid

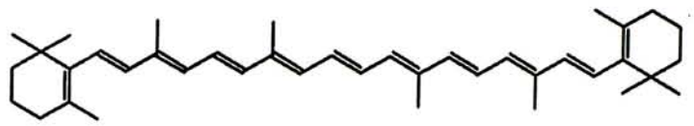


13 - *cis* - retinoic acid

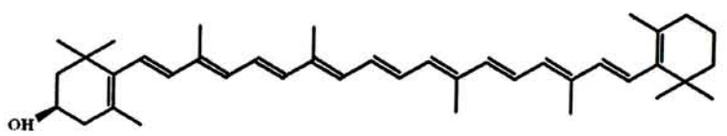
Provitamin A - carotenoids:



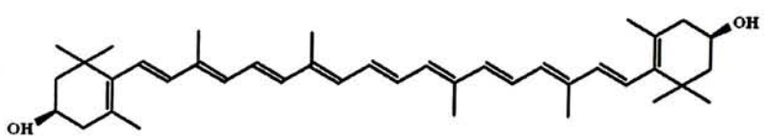
α- carotene



β- carotene



β- cryptoxanthin



Zeaxanthin

Figure 2.10 Chemical structures of vitamin A-active retinoids and common pro-vitamin A carotenoids

2.4.2 Roles of vitamin A and carotenoids in diseases prevention

Reports of correlation between intake of vitamin A or carotenoids containing diet with health benefits have been recorded as early as 1970s. It has been proven that vitamin A is involved in varies cellular mechanisms, for instance, the proper functioning of optical sensing mechanism of the visual cycle. Also, the antioxidant activity of carotenoids is suggested to reduce the incidence of chronic diseases. Recent studies have also shown a positive correlation between several carotenoids and certain types of cancers.

2.4.2.1 Visual cycle and related diseases

Long-term consumption of vitamin A - deprived diet would lead to night-blindness, indicating the important role of vitamin A for vision under dim light situation. Vitamin A involved in light detection is in the form of 11-*cis*-retinal, which acts as a light-absorbing component associated with opsin. This 11-*cis*-retinal:opsin complex is known as rhodopsin located at the outer segments of rod photoreceptor cells on the retina. Once light stimulates the photoreceptors, the 11-*cis*-retinal is isomerized into all-*trans*-retinal and dissociated from the opsin. Such change in the conformation of rhodopsin activates transducin which subsequently initiates a change of potential across the cell membrane leading to a cascade of signal transduction along the neurone, the optic nerve, towards the brain. The deactivated opsin re-attaches with another 11-*cis*-

-retinal to form rhodopsin and regain its sensitivity. If opsin remains detached from 11-*cis*-retinal, vision under dim light condition would be lost.

The 11-*cis*-retinal required to activate opsin comes from three ways. First, there is a supply of 11-*cis*-retinal directly from the circulation to the retinal pigment epithelium. The other way involves the recycle of the all-*trans* isomer. The all-*trans*-retinal dissociated from opsin is reduced into all-*trans*-retinol, and is escorted by the interphotoreceptor retinoid-binding protein to the retinal pigment epithelium. After being esterified, hydrolyzed, isomerized and oxidized, the all-*trans*-retinol is converted back into 11-*cis*-retinal, which is finally released to the rod outer segment, where it can re-attach with opsin forming rhodopsin (Lamb and Pugh, 2004, and Pepperberg and Crouch, 2001)

It is also reported that age-related eye diseases such as cataract and macular degeneration can be prevented by dietary carotenoids, such as lutein and zeaxanthin, which help quenching reactive oxygen species that attack the ocular tissues (Brown *et. al.*, 1999).

2.4.2.2 Cardiovascular diseases

Previous studies have shown the correlation between carotenoids and the development of cardiovascular diseases. It is suggested that the antioxidant activity is the key factor for the preventative potential of carotenoids (Kohlmeier and Hastings,

1995). The consumption of anti-oxidizing carotenoids such as lycopene can reduce oxidative damage of lipoprotein. Similar prevention of oxidation of lipids in plasma and plaque which may cause blockage of blood vessels is also reported (Hadley *et. al.*, 2003).

2.4.2.3 Cancer

It has been reported that carotenoids show properties against cancer formation, such as protecting genome stability, inhibiting cell proliferation, transformation and micronucleus formation (Collins, 2001). Further studies reported a positive effect of carotenoids in certain types of cancers.

It has been shown that high lycopene level was associated with reduced risk of breast cancer in which lycopene inhibits cell cycle progression at the growth phase (Nahum *et. al.*, 2001) while lutein was inversely associated with the incidence of colon cancer (Slattery *et. al.*, 2000). In 1990s, studies have been carried out and proved that carotenoids can reduce the incidence of erythema. A mixture of β-carotene and vitamin E in diet can also be beneficial in preventing the development of skin cancer (Stahl *et. al.*, 2000). In vitro study also proved that addition of β-carotene, lutein and lycopene can reduce UV-induced lipid peroxidation of human skin fibroblast cells (Eichler *et. al.*, 2002). A high accumulation of lycopene in prostate after tomato sauce supplementation led to studies on the correlation between lycopene and prostate cancer. It is found that

intake of high tomato diet can lower the risk of prostate cancer (Barber, 2003). Also, in patients diagnosed with prostate cancer, a high tomato diet can help reducing leukocyte oxidative DNA damage and prostate tissue oxidative damage (Chen *et. al.*, 2001).

2.4.3 Roles of vitamin A in gene regulation

Studies have reported the regulatory effect of vitamin A on gene transcription. It is found that all-*trans* and 9-*cis* retinoic acids bind to and activate retinoic acid and retinoid X receptors respectively. These receptors then recognize and bind onto the retinoic acid response elements (RAREs) on the DNA and finally initiate the gene transcription. Up to now, over 500 genes are proposed to be regulated by vitamin A either under direct control of RAREs or other indirect regulatory mechanism (Balmer and Blomhoff, 2002). Also, it is suggested that by controlling gene transcription, vitamin A poses regulatory effects on immune defence, tissue epithelialization, morphogenesis, organogenesis, bone growth and development (Blomhoff and Blomhoof, 2006 and Zile, 2001).

2.4.4 Bioavailability and daily intake recommendation

As described in previous session, vitamin A is necessary for maintaining the proper functioning of many physiological mechanisms of our body, hence, maintaining our health, and is obligatorily provided from the diet. Therefore, we have to meet the

dietary vitamin A requirements, or recommended daily allowance (RDA) of vitamin A.

Vitamin A activity is measured based on that of 1μg of retinol, which is known as 1μg of retinol activity equivalent (RAE). According to the US RDA of vitamin A, people of different ages, gender and at different states may have different requirements. For example, the RDA for children aged 1 -3 years old is 300μg RAE per day. As for adults, the RDA are 900μg RAE and 700μg RAE for male and female respectively. A pregnant woman requires a relatively higher RDA about 760μg RAE. These RDA can be fulfilled by consumption of preformed vitamin A, in retinal, retinol, retinoic acid or in the ester form, from animal source. Typically, 100 gram of liver provides 5000 to 12000μg RAE, while whole milk and cheeses provide 30 - 60μg RAE and 100 - 200μg RAE respectively.

Provitamin A carotenoids from plant sources, such as dark green spinach, yellow flesh mango and papaya, are also dietary sources of vitamin A and are the major sources for the developing countries. However, the bioconversion and bioavailability of carotenoids from plant sources have to be considered when we estimate their RAE. Based on the study by the Institute of Medicine in the United States in 2001, the bioconversion factors of β-carotene and other provitamin A cartotenoids to retinol equivalent are 12:1 and 24:1 respectively (Otten *et. al.*, 2006). The conversion factor of β-carotene is higher, and may reach about 21:1, for the malnutrition population (van

Lieshout and de Pee, 2005).

Apart from bioconversion, bioavailability is another important factor influencing the amount of vitamin A equivalent provided by provitamin A carotenoids. Bioavailability is defined as the amount of ingested nutrient available to the body for physiological functions or for storage (Jackson, 1997). There are nine factors affecting the bioavailability of carotenoids and are summarized in the mnemonic SLAMENGHI (Castenmiller and West, 1998). These factors include the **S**pecies of carotenoid and its molecular **L**inkage, the **A**mount of carotenoid eaten and the **M**atrix in which the carotenoids is taken with, the **E**ffectors of absorption and bioconversion, the **N**utrient states, **G**enetic make-up and **H**ealth status of the host, and finally the **I**nteractions with other nutrients; for example, for β-carotene, all-*trans*-β-carotene is absorbed better than the 9-*cis* isomer; cooking methods such as mincing and pureeing can improve bioavailability, 5 - 10 gram of fat in meal can improve bioavailability, low protein and zinc status of the host reduces β-carotene, the absorption of β-carotene would be reduced for host suffered in intestinal and malabsorptive diseases, and finally, supplementation of one carotenoid may affect the plasma concentration of the other (West and Castenmiller, 1998 and de Pee and West, 1996). Therefore, more researches have to be taken to determine the RAE of specific carotenoids from different food sources in order to estimate whether the RDA could be met.

2.5 Vitamin A deficiency (VAD)

Vitamin A deficiency is a global health problem affecting more than 118 countries worldwide, and is considered as a serious public health problem in at least 26 countries including highly populated area in Asia, Africa and Latin America(Ye *et. al.,* 2000), as shown in Figure 2.11.

Most of these suffered populations are under-nourished in regions with scarce food sources, limited food diversity and poor infrastructure making the delivery of supplements difficult, or even impossible.

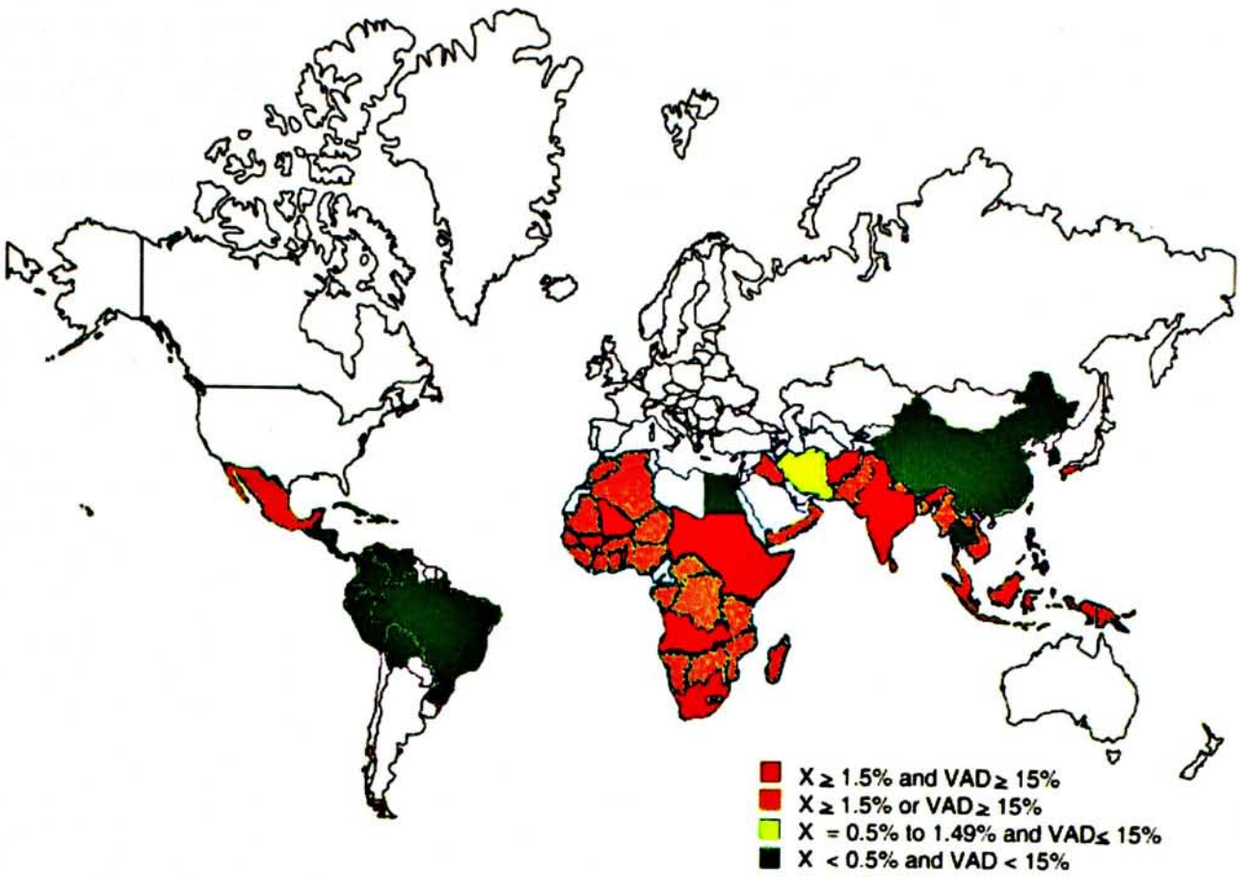


Figure 2.11 Global distribution of preschool-child suffered from vitamin A deficiency (VAD) (re-drawn from West, 2002)

VAD defined by joint prevalence of deficient serum retinol concentration (<0.70μmol/L) or abnormal conjunctival impression cytology and xerophthalmia (X)

2.5.1 Clinicopathological features

Since vitamin A is involved in the proper functioning and maintenance of vision, immune response, bone growth, reproduction, surface linings of eyes and the epithelial integrity, deficiency of this micronutrient would lead to a series of clinical symptoms.

2.5.1.1 Visual problems

Night blindness, as described in previous session, is the earliest symptom. Prolonged deficiency would result in drying of conjunctiva, which may then extend to the cornea, known as xerophthalmia. If the condition is not relieved, the impaired cornea would eventually shrivelled and ulcerated, leading to a clinical feature known as keratinomalacia. Finally, inflammation and infection may occur in the interior of eye resulted in total and irreversible blindness (Al-Babili and Beyer, 2005).

VAD affects 140 - 250 millions preschool children globally, and is the primary cause of childhood blindness, causing xerophthalmia in 5 millions children every year, in which at least 0.25 million go eventually blind and half of them die within 12 months of losing their sight.

Besides, VAD also affects pregnant women who may suffered from night blindness in the latter half of pregnancy at which a higher nutrition demand is needed (Christian *et. al.,* 1998).

2.5.1.2 Infection

Since vitamin A is important for immune system, VAD increases morbidity and mortality in children by depressing the specific and non-specific immune mechanism (Datta *et. al.,* 2003). On the other hand, lack of vitamin A may lead to poor repair and maintenance of epithelial linings. This results in a loss of barrier and makes those suffered from VAD vulnerable to infection.

As for the early infancy, due to the limited retinol supplied from materno-fetal transfer, the low retinol concentration in blood increases the risks of morbidity and mortality in the first 6 - 12 months of life.

It is also found that children suffered from VAD shown increased susceptibility to potentially fatal afflictions such as diarrhoea, respiratory disease and childhood diseases like measles (Burkhardt *et. al.,* 1997). Thus, VAD is a major contributing factor of 1 - 3 millions child deaths each year.

On the other hand, VAD may lead to other disorders, for instance, impaired iron mobilization (Al-Babili and Beyer, 2005); thus, VAD is found to contribute indirectly to anaemia by interfering with iron bioavailability (Schaub *et. al.,* 2005).

2.6 Global efforts in combating VAD

Supplementation of vitamin A or provision of improved vitamin A diet can help alleviating VAD. It is found that a diet rich in vitamin A can reduce 1 - 2 million deaths

of children aged 1 - 4 years old, and prevent 0.25 - 0.5 million deaths during later childhood (Humphrey *et. al.,* 1992). In Yemen, after a year of semi-annual vitamin A supplementation, the case of admissions and fatality due to dehydrating diarrhoea of preschool children were reduced by 25% and 50% respectively (Banajeh, 2003). It is also reported that there are about 60000 women died from childbirth-related caused every year, and would be reduced if vitamin A enriched diet is provided (Al-Babili and Beyer, 2005). A once-a-week supplementation with either vitamin A or β-carotene approximately of the RDA can help reducing pregnancy-related mortality by 40% and 49% respectively (West *et. al.,* 1999).

Therefore, global efforts have been made to target at increasing vitamin A content in diet or providing vitamin A supplementation to alleviate VAD in the developing countries. These measures include dietary diversification, supplementation and fortification.

2.6.1 Dietary diversification

The aim of dietary diversification is to increase the variety of food that is affordable and stably supplied so that those in need can have a diverse food source of vitamin A. Global efforts have been made in different aspects to achieve the goal.

In order to alleviate VAD for infants, a high-dose of vitamin A supplement is given to postpartum women within 6 weeks after delivery (Ross, 2002) and at the same time,

promote and extend breast-feeding through education. This campaign helps to increase the retinol level in breast milk for 2 - 8 months after maternal postpartum (Rice *et. al.*, 2000), hence, more vitamin A can be delivered to the infants. On the other hand, supply of soft yellow fruits, dark green vegetables and egg is provided to preschool children routinely. This can help compensating the relatively less nutritious breast milk of the undernourished populations.

Homestead gardening is also promoted, so that the needy can provide themselves sustainable food source and improve household income (Talukder *et. al.*, 2000). By this mean, they can enjoy a long-term improvement of food security and diversity. Besides, food diversity is extended by promoting the consumption of red and black landraces of rice, orange fruits, root and tuber, red palm oil and animal sources with high vitamin A level such as liver and meat (Stein *et. al.*, 2008).

Finally, since the bioavailability of provitamin A carotenoids could be affected by the method of cooking, mincing and pureeing are recommended and promoted.

The limitations of this preventive method include the lack of infrastructure needed to reach the remote rural region to start education and promotion. Also, a continuous and sustainable funding is needed to provide routine food supply to the needy. And it is hard to change one's eating habit, for example, it could be difficult to promote the consumption of meat, liver and other animal products in a society with many

vegetarians; or it is hard to promote the consumption of fruits and vegetables, in a region where these food are seasonal and expensive, to families with limited income (Stein *et. al.*, 2008).

2.6.2 Supplementation

Vitamin A supplementation is another traditional intervention employed to combat VAD. In recent decades, a periodic high-dose vitamin A in capsule or oily syrup is routinely provided to preschool children, pregnant and postpartum women. Since vitamin A absorbed can be stored in the liver, therefore, once in need, it can be released to meet the body demand and sustain the plasma retinol level for a period of time. Every year, UNICEF provides more than 400 millions vitamin A supplements to about 80 countries. With the co-operation from local clinic and health centre distributed in rural area and villages, vitamin A supplements can reach children even in the remote region.

However, there are still limitations on extending the coverage, especially for reaching remote region with poor infrastructure. Also, the maintenance of adequate distribution logistics, the way to identify and target the vulnerable populations, requirement of on-going costs for sustaining the campaign, minimal health personnel training and joint support by the government may hinder the supplementation programme from extending further (Al-Babili and Beyer 2005 and Stein *et. al.*, 2008).

2.6.3 Pro-vitamin A enriched crops by genetical engineering

2.6.3.1 Tomato

Due to the high carotenoids content in tomato fruit, it and also its food derivatives have long been a major source of lycopene and β-carotene in human diet, especially in the western countries. Lycopene is the most abundant carotenids in ripening tomato, accounting for about 90% of total fruit carotenoid. During fruit ripening, there is a rapid formation of lycopene accompanied by up-regulation of carotenogenic enzymes PSY and PDS and a down-regulation of LCY-b which converts lycopene into β-carotene. With such a high level of substrate, lycopene, for β-carotene production, tomato is one of the major targets in biotechnology and several attempts have been carried out aiming at enhancing the β-carotene content.

Since the rate-limiting step is the phytoene production by PSY, a bacterial phytoene synthase (CrtB) linked to a transit peptide from pea small subunit of RUBISCO was expressed specifically in fruit. The resulted fruit shown a 2 - 3 fold of increase in carotenoids. Other attempts focused on PDS. A bacterial phytoene desaturase (CrtI) was expression fruit-specifically and was targeted to plastid with the use of transit peptide from pea small subunit of RUBISCO. Although the transgenic fruit shown 1.6 - 2.4 fold increase in β-carotene and 1.8 - 2.1 fold in lycopene, fulfilling half RDA of provitamin A by a single fruit, amount of prior carotenoids decreased (Fraser *et. al.*,

2002). Further study on other plant's carotenogenic enzymes was carried out. The expression of the *Arabidopsis* LCY-b under the tomato PDS promoter led to a increase of fruit β-carotene by 5 fold, without affecting the amount of other carotenoids in fruits (Fraser and Bramley, 2004).

2.6.3.2 Potato

Potato is one of the world important calories sources; thus, nutritional improvement in the potato tuber would bring great impact. Due to the low carotenoids content, potato tuber is also a target for genetical engineering. Under the control of the tuber-specific promoter, a bacterial CrtB was expressed in the white-flesh *Solanum tuberrosum* L. and the yellow-flesh *Solanum phureja* L. respectively. The results were promising, the total carotenoids was increased from 5.6μg/g to 35μg/g of white-flesh tuber; and from 20μg/g to 78μg/g of yellow-flesh tuber (Ducreux *et. at.*, 2005). Owning to the physiological differences in tuber and fruits, the expression of CrtB in potato did not lead to any abnormal change in tuber ABA level, which has been reported in transgenic tomato.

2.6.3.3 Canola

The attempt of enhancing pro-vitamin A in canola achieved the greatest success. Originally, there is negligible β-carotene in canola seed. The seed-specific expression of

bacterial CrtB led to a 50 fold increase in carotenoids and a 300 fold β-carotene enhancement (Shewmaker *et. al.*, 1999). In the orange embryo, increases levels of α-carotene and β-carotene were detected, and several new carotenoids species such as phytoene and lycopene were also found. Though 50% decrease in tocopherol levels and 80% reduction in chlorophyll level during seed maturation were observed, the high boost in β-carotene was promising.

2.6.3.4 The Golden Rice (GR) project

Fortification of existing food with vitamin A is a preventive measure for alleviating VAD. This measure required a central process on food items. Up to now, various fortified food are distributed in different countries, for example, vitamin A fortified sugar in Central America and fortified monosodium glutamate in Southeast Asia, other vitamin A fortified food include margarine, wheat flour and milk powder.

Since rice is a major food staple for more than 2 billions people worldwide and account for 80% daily calories intake in Western Pacific and Southeast Asia, where VAD is affecting 14% and 33% of preschool child under 5 years old respectively (West, 2002), it is a target of vitamin A fortification. However, as there is no naturally-occurring rice variety with provitamin A carotenoids in the endosperm, traditional breeding cannot be employed to make vitamin A fortified rice. Instead, due to the recent advance in molecular biotechnology and the long history of elucidation on

plant carotenogenesis, rice enriched with provitamin A carotenoids can be made by genetic engineering. This breakthrough is known as the Golden Rice project.

2.6.3.4.1 The 1st generation (GR2)

Because the carotenoid biosynthesis pathway in rice endosperm is incomplete, it does not accumulate any carotenoids, but the precursor GGPP. By filling the gaps with foreign carotenogenic enzymes, scientists have successfully made the 1st generation of Golden Rice (GR1) which accumulated yellow β-carotene in the endosperm (Ye *et. al.*, 2000), as shown in Figure 2.12.

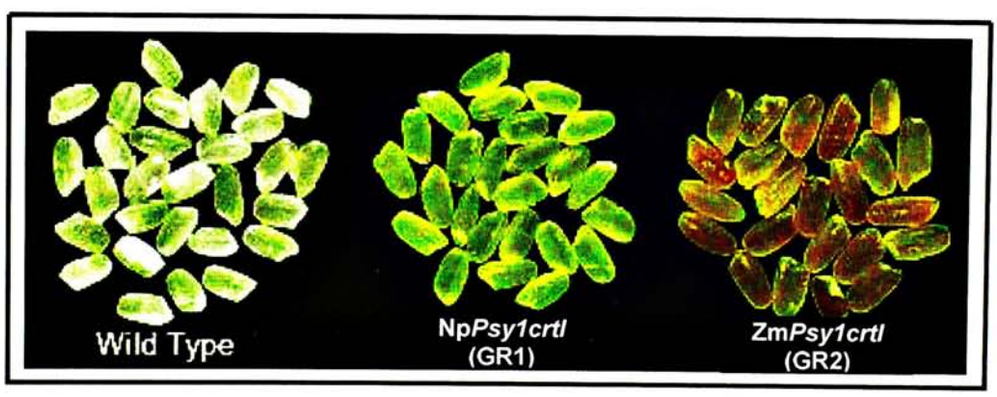


Figure 2.12 Pictures of polished wild-type and transgenic rice seeds, transformed with *Narcissus pseudonarcissus(Np) psy* and *Zea mays(Zm) psy1* respectively (adopted from Paine *et. al.*, 2005)

Transgenic grains are yellow to orange with the colour intensity corresponding to the carotenoids content in endosperm; in comparison with the white wild type grain

In this project, in order to complete the carotenogenesis pathway in rice endosperm, a PSY gene from *Narcissus pseudonarcissus* was expressed under the seed-specific Gt-1 promoter; while a bacterial CrtI gene from *Erwinia uredovora* with a transit peptide from pea RUBISCO small subunit was constitutively expressed with a *CaMV35S* promoter. This bacterial CrtI enzyme was able to substitute plant's desaturases, PDS and ZDS, and isomerase, ZISO and CrtISO, to convert phytoene into all-*trans*-lycopene. The co-expression of these two foreign genes led to an accumulation of 1.6μg β-carotene per gram of dry endosperm. The yellow endosperm indicated the accumulation of this carotenoid.

In the transgenic rice, the reaction did not stop at accumulation of lycopene as expected, instead, it went further to produce β-carotene. It was because there were relatively weak expressions of the endogenous LCY-b and hydroxylase in rice endosperm (Schaub *et. al.,* 2005), which converted the lycopene formed into β-carotene.

Since the content of β-carotene was relatively low so that a large amount of GR1, several hundreds grams, would be needed to fulfill the RDA of vitamin A; thus, further studies were carried out to improve the β-carotene content in the rice endosperm.

2.6.3.4.2 The 2nd generation (GR2)

In order to enhance the β-carotene content in transgenic rice endosperm, scientists had to find out the limitation that hinder β-carotene production in GR1. Since there was

no phytoene accumulation in GR1, the activity of the bacterial CrtI was not the limiting step; instead, the PSY from *Narcissus pseudonarcissus* may be the target.

Due to the rate-limiting property of PSY for carotenoids biosynthesis in some wild-type tissues, PSY was considered as a major regulatory step in the pathway. Therefore, Paine *et. al.* (2005) tried to study the enzymatic activities of PSY from various plant species, for instant, pepper, tomato, rice and maize using the maize calli. It was found that the enzymatic activities of rice and maize PSY were much higher than that of the *Narcissus pseudonarcissus* gene used in GR1.

Since maize psy was known to be functional in maize endosperm for carotenoids production, forming the yellow endosperm rich in zeaxanthin, it was expressed seed-specifically together with the bacterial CrtI to produce the 2nd generation of Golden Rice (GR2). The use of maize PSY resulted in a boost of β-carotene up to 37μg β-carotene per gram of dry endosperm with a distinct orange endosperm, shown in Figure 2.12.

Assuming the bioconversion factor of β-carotene available in GR2 to retinol equivalent is 12:1, and that an individual receives at least some vitamin A from other sources, 72 grams of GR2 per serving would be able to provide 180μg RAE, which is about 50% of the RDA of a 1 - 3 years old children. Further follow-up experiments have been conducted to determine the actual bioconversion factors of GR2. It was concluded

that the bioconversion of β-carotene from GR2 was around 6:1, which was independent from the sex and BMI of the volunteers. This high conversion efficiency was suggested to be contributed by the simple food matrix of cooked rice consumed together with 10 grams butter. Thus, for GR2 with 20 - 30μg of β-carotene per gram of dry endosperm, only about 100 grams of uncooked rice can provide 500 - 800μg RAE, which was equivalent to 55 - 70% of the RDA for adult (Tang *et. al.,* 2009).

With such an efficient bioconversion, GR2 was a possible tool for combating VAD in the deficient countries.

2.7 Rice phytoene synthase as a GR candidate enzyme

2.7.1 General properties of phytoene synthase in higher plant

As described in Section 2.2.2, phytoene synthase is the enzyme catalyzing the first committed step of carotenogenesis forming the first colourless carotenoid phytoene by a condensation of two GGPP. It is also found to be the rate-limiting step for carotenoid biosynthesis in tomato fruit (Fraser *et. al.,* 1994) and *Arabidopsis* seed (Lindgren *et. al.,* 2003).

With the advance in molecular genetics, a number of phytoene synthase genes have been cloned and characterized from different high plant species for instance, *Narcissus pseudonarcissus* (daffodil), *Arabidopsis thaliana*, *Capsicum annuum* (pepper),

Helianthus annuus (sunflower), *Tagetes erecta* (Mexican marigold), *Cucumis melo* (huskmelon), tomato, maize and rice.

2.7.1.1 Gene duplication and structure

Most of the dicot plants have only one phytoene synthase gene (*psy*) in their genome, including daffodil (accession no. P53797), *Arabidopsis* (accession no. NP_197225), pepper (accession no. P37272), sunflower (accession no. CAC19567), Mexican marigold (accession no. AAM45379) and huskmelon (accession no. P49293); whereas, multiple copies of phytoene synthase genes have been identified in the genome of tomato, an exception for dicot plants, and most monocot plants, especially members of the *Poaceae* family. There are two *psy* for tomato, *Slpsy1* (accession no. EF534740) and *Slpsy2* (accession no. EF534738), and three copies for maize, *Zmpsy1* (accession no. AAR08445), *Zmpsy2* (accession no. AAX13807) and *Zmpsy3* (accession no. ABD1701).

As for the gene structure, although the lengths of *psy* cDNA of different species are different, all of the *psy* from maize, tomato, *Arabidopsis* consists of five introns and six exons of similar size (Giorio *et. al.,* 2008 and Li *et. al.,* 2008a).

When comparing the amino acid sequence of maize *psy1* to those of pepper, *Arabidopsis*, tomato *psy1*and *psy2*, similarities ranging from 78 - 81%, and an identity from 67 - 71% were observed (Buckner *et. al.,* 1996).

2.7.1.2 Membrane association and cation requirement

It has long been noted that carotenoid biosynthesis takes place on the membranes of chloroplast, chromoplast, amyloplast, elaioplast and leucoplast (Gallagher *et. al.*, 2004; Kirk and Tiliney-Bassett, 1978; Janick-Buckner *et. al.*, 1999; Shewmaker *et. al.*, 1999 and Parry and Horgan, 1992). Characterization of daffodil *psy* revealed that phytoene synthase existed in two forms in the chromoplast stroma: the inactive soluble form and active membrane-bound form, suggesting a requirement of membrane association for enzymatic activity. Biochemical assays have been done to show that phytoene synthase interacts hydrophobically with membrane but not as an integral membrane protein. Besides, phytoene synthase exhibited galactolipid association, and substitution by other sugars would reduce activity, suggesting that galactose was required for catalyzing the enzymatic activity.

On the other hand, the daffodil *psy* also exhibited an absolute requirement for Mn^{2+} ion, substitution by other bivalent cations led to great reduction in activity (Schledz *et. al.*, 1996). Similar phenomenon has been reported suggesting a strict dependence of Mn^{2+} ion even as high as 4mM for pepper *psy* activity. Also, pyrophosphate was suggested to be an inhibitor for *psy*, as it bound to the substrate's active site on the enzyme (Dogbo *et. al.*, 1988).

2.7.1.3 Expression pattern and tissue-specificity

Gene duplication of phytoene synthase has aroused scientists' interest to elucidate the expression pattern and activity of each homolog in different tissues. Here, studies conducted in tomato and maize will be discussed.

In tomato's genome, it has been suggested that there is one fruit *psy* copy (Bartley *et. al.,* 1993) and one green organ and flower copy (Fray and Grierson, 1993). Quantitative RT-PCR has been carried out with total RNA isolated from various wild type tissues of tomato. The expression patterns suggested that *Slpsy1* was expressed predominantly in the coloured petal, anther, developing and ripen fruits while *Slpsy2* showed a higher expression level in the root, leaf, sepal and ovary. These suggested that *Slpsy1* was responsible for phytoene formation in chromoplast while *Slpsy2* was chloroplast-specific (Giorio *et. al.,* 2008). Similar conclusion has been drawn when studying the yellow flesh *r,r* mutant and the constitutively expressed *Slpsy1* antisense mutant. In these two mutants, only *Slpsy1* expression was down-regulated while that of *Slpsy2* remained normal as proved by RT-PCR. Fruits obtained from these mutants were yellow-flesh suggesting a reduction in red lycopene production which cannot be rescued by *Slpsy2* activity even though expression of *Slpsy2* was detected. As for the green tissues, although there was a reduction in total carotenoid, the carotenoid to chlorophyll ratio has not been affected even deprived in *Slpsy1* expression. This suggested that *Slpsy2* expressed in green tissue can compensate the *Slpsy1* activity and maintain a

normal carotenoid to chlorophyll ratio (Fraser *et. al.*, 1999).

As for the studies on maize, maize endosperm is rich in lutein and zeaxanthin which confers the endosperm yellow. A maize *psy1* mutant shown pale-yellow endosperm with normal green leaves, suggesting the presence of another *psy* copy in the maize's genome responsible for leaf-specific activity (Buckner *et. al.*, 1996). Recently, all of the three maize *psy* copies have been cloned and characterized. In wild type tissues, though, transcripts of the three maize *psy* can be detected in leaves and root, action of *Zmpsy1* was suggested to be endosperm-specific (Gallagher *et. al.*, 2004); *Zmpsy2* was leaf-specific, while *Zmpsy3* was root-specific and was thought to be responsible for carotenoid biosynthesis essential for ABA biosynthesis induced by abiotic stresses (Li *et. al.*, 2008a). Further study has also demonstrated the localization of *Zmpsy1* on the envelop membrane of amyloplast. Since chloroplast contains both the thylakoid and envelope membranes, it is suggested that two carotenoid biosynthetic pathways may present in chloroplast. Besides, knock-out *Zmpsy1* mutant showed a reduction in carotenoid level in dark and suffered from severe photobleaching at elevated temperature in light which could not be rescued by the action of *Zmpsy2*. These suggested the importance of *Zmpsy1* for carotenoid production in dark and under heat stress (Li *et. al.*, 2008b).

2.7.2 Rice phytoene synthases: *Ospsy1, Ospsy2 and Ospsy3*

2.7.2.1 Gene duplication and structure

There are three copies of *psy* identified in the rice's genome named as *Ospsy1* (accession no. AJ715786), *Ospsy2* (accession no. AK073290) and *Ospsy3* (accession no. NM_001070427). All of the three rice's homologs consist of five introns and six exons similar with the *psy* orthologs identified in other higher plants (Welsch *et. al.,* 2008).

2.7.2.2 Protein structures and phylogenetic analysis

The three rice *psy* homologs *Ospsy1, Ospsy2* and *Ospsy3* encode for a functional enzyme with phytoene synthase activity with the size of 42kDa, 40kDa and 43kDa respectively. The amino acid sequences of these homologs share 68% of similarity and 58% of identity. Plastid transit peptides have been predicted with ChloroP with 21, 80 and 53 amino acids respectively and all of them are membrane associated.

Comparison of rice's PSY with PSY orthologs from other higher plants suggested that they are grouped together with maize PSY and daffodil PSY in a separated branch of monocot PSYs (Welsch *et. al.,* 2008), as shown in Figure 2.13.

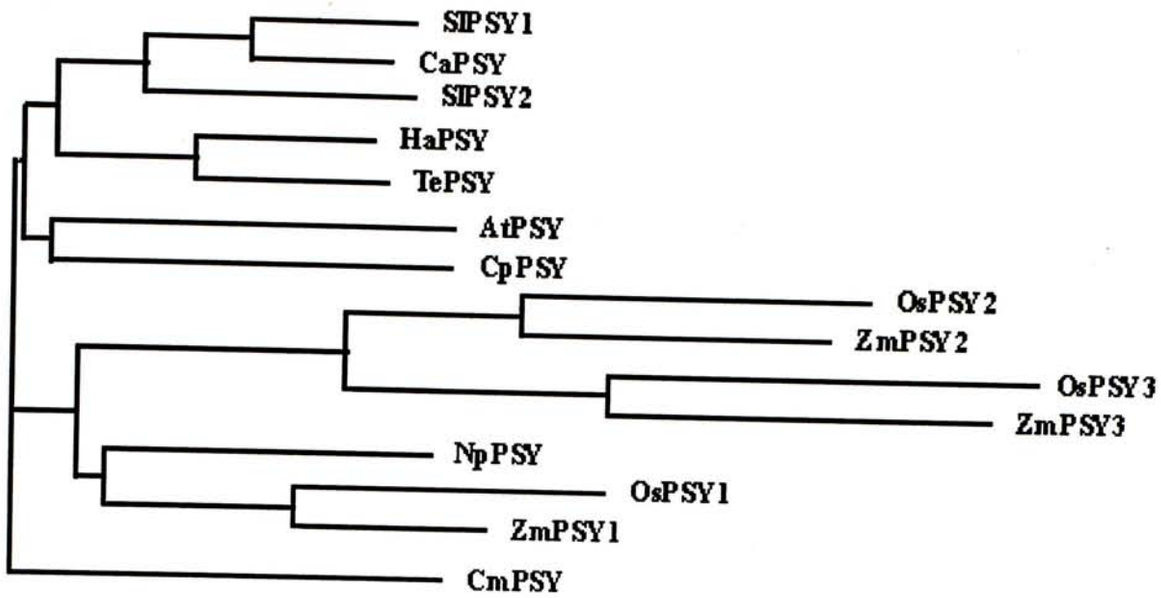


Figure 2.13 Phylogenetic tree diagram of rice PSYs and selected plant PSYs (adopted from Welsch *et. al.*, 2008)

A phylogenetic comparison of PSY amino acid sequences of selected plants. Abbreviations and GeneBank accession numbers are listed below: AtPSY (*A. thaliana*; NP_197225), CaPSY (*C. annuum*; P37272), CmPSY1 (*C. melo*; P49293), CpPSY (*Citrus* x *paradisi*; AAD38051), HAPSY (*H. annuus*; CAC19567), NpPSY (*N. pseudonarcissus*; P53797), OsPSY1 (*O. sativa*; AJ715786), OsPSY2 (*O. sativa*; AK073290), OsPSY3 (*O. sativa*; NM_0010170427), SlPSY1 (*S. lycopersicum*; EF534740), SlPSY2 (*S. lycopersicum*; EF534738), TePSY (*T. erecta*; AAM45379), ZmPSY1 (*Z. mays*; AAR08445), ZmPSY2 (*Z. mays*; AAX13807) and ZmPSY3 (*Z. mays*; ABD1761).

2.7.2.3 Expression pattern

In wild type rice tissues, real time PCR revealed the expression of the three *Ospsys* in leaves with that of *Ospsy1* being the highest. Although all of the three rice *psy* were expressed in root, the expression levels were very low. In the immature endosperm, transcripts of none of them can be detected (Welsch *et. al.,* 2008)

These results contradict with the findings by Gallagher *et. al.* (2004), who had reported the expression and translation of *Ospsy2* in mature endosperm.

The expression profiles of transcripts in leaves and developing seeds of both *Ospsy1* and *Ospsy2* in wild type *Japonica* 9983 rice had been elucidated by northern blot which showed the expression of *Ospsy1* in leaves but absence in the seeds throughout seed maturation (Figure 2.14A). In contrast, transcript of *Ospsy2* was detected in leaves and at early stage of seed development. The expression of *Ospsy2* was the highest in immature seed collected 5DAF, and the transcript level was much lower and kept in a constant level at later stages, from 10DAF to 20DAF, (Figure 2.14 B), (Leung and Sun, unpublished data).

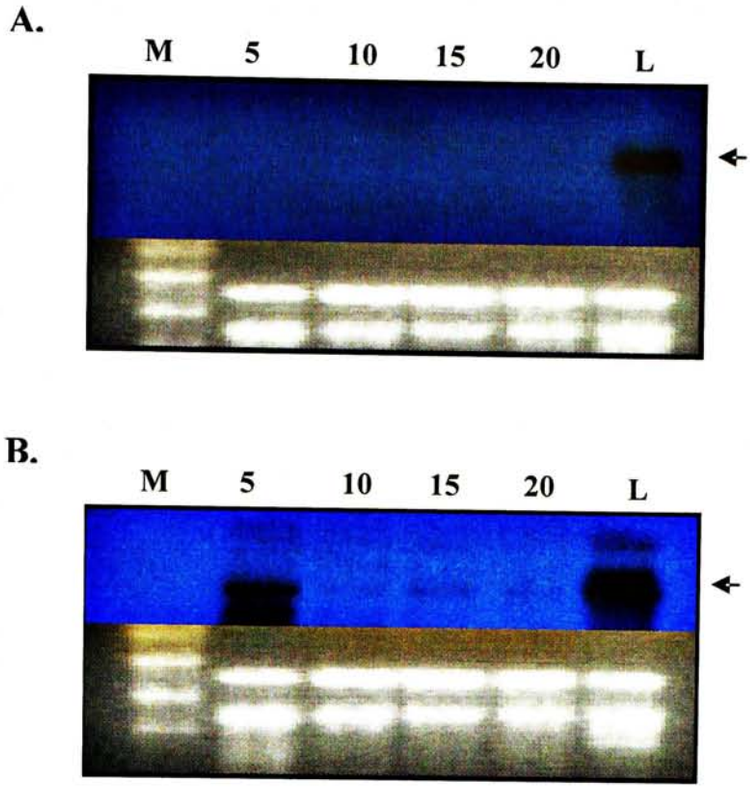


Figure 2.14 Expression profiles of *Ospsy1* and *Ospsy2* in wild type rice tissues
(Leung and Sun, unpublished data)
Expression profiles of *Ospsy1* (A) and *Ospsy2* (B) in different rice tissues of *Japonica* 9983. Lanes: M, 0.16 - 1.77kb RNA ladder (Invitrogen); 5, 10, 15 and 20, wild type immature seeds sampled at development stage at 5, 10, 15 and 20 DAF respectively; and L, wild type leaves samples. Expected sizes of both *Ospsy* are 1.2kb as indicated by arrow.

2.7.2.4 Light- and stress-induced expression

The expression of *Ospsy1*, *Ospsy2*, and *Ospsy3* under different radiations and abiotic stresses has been elucidated.

It was found that the expression of *Ospsy1* and *Ospsy2* could be up-regulated by the exposure to red, far red and white radiations. This was consistent with the presence of light-responsive elements, including the BoxI, BoxN and AE Box elements, in the promoters of *Ospsy1*and *Ospsy2* whereas *Ospsy3* was not light-regulated. It was also

interesting to note that *Ospsy1* expression could be detected in dark which might be responsible for carotenoid production in the etioplast which is important for chloroplast formation.

When subjected to abiotic stresses, for instance, drought, salt and ABA treatments, up-regulation of *Ospsy2* in leaves and *Ospsy3* in both leaves and root were detected. Since the promoter of *Ospsy3* contains an ABA-responsive element, it is suggested that *Ospsy3* is the primary and secondary responder to stresses. Under stressed conditions, it acts as a primary responder to increase carotenoid production which is the precursor for ABA biosynthesis in root. Later, ABA is delivered from root to leaves by root-to-shoot ABA transport via xylem to induce *Ospsy3* expression in leaves, which further enhances ABA production in leaves (Welsch *et. al.,* 2008).

2.7.3 Rice phytoene synthases activity in rice seeds

2.7.3.1 Previous study in rice seed carotenogenic capacity

Biochemical analysis has been done to verify the carotenogenic capacity of rice endosperm. Work by Kleinig (1989) has demonstrated the presence of GGPP in rice endosperm which is believed to be localized in the plastid. In vitro incubation of wild type immature endosperm without seed coat in the presence of radiolabelled [1-^{14}C]IPP showed no production of any carotenoids, but an accumulation of radioactive GGPP,

suggesting the GGPP synthetic activity of the immature rice endosperm. Incubation of the same immature endosperm with radiolabelled [^{14}C]GGPP and [^{14}C]phytoene showed no production of carotenoids, suggesting that there was no carotenogenic capacity in rice endosperm. Even though expressions of endogenous PDS, ZDS, ZISO, CrtISO and LCY-b were detected inside the rice endosperm, the absence of functional PSY and weak enzymatic activities of the expressed endogenous genes made it impossible for carotenoids production in rice endosperm. On the other hand, trace amounts of phytoene was detected in isoprenoid extracts from wild type mature seed without removal of seed coat by HPLC analysis. Since the rice endosperm lacked the ability to produce phytoene, it was suggested that the rice seed coat, a photosynthetically active tissue prior to seed maturation, was responsible for the phytoene detected (Burkhardt *et. al.,* 1997).

Because rice is milled to remove the oil-rich pericap, seed coat and aleurone layer during processing so as to prevent it from turning rancid upon storage especially in tropical and subtropical areas, the milled rice consumed by human lacks any carotenoids.

2.8 Seed-specific rice promoters

2.8.1 Previous studies on seed-specific expression in rice

Rice is a major staple food and an important dietary protein source for more than 40% of the world's population. In general, rice seed storage proteins can be classified into four groups. They are the insoluble glutelins, alcohol-soluble prolamins, salt-soluble globumins and water-soluble albumins (Wu *et. al.,* 1998a). Since the expressions of these storage proteins are confined in the rice seed, the promoters directing their seed-specific expression arouse much research interest.

Study has been conducted to verify the expression pattern directed by the promoters of different rice seed storage protein genes using GUS as a reporter. It was found that the glutelin A gene promoters, GluA-1, GluA-2 and GluA-3 direct specific expression in the outer endosperm while the glutelin B gene promoters, GluB-1, GluB-2 and GluB-4 direct expression in the aleurone, subaleurone and the outer endosperm. In contrast, GluB-5 and GluC promoters are specific to the whole endosperm.

The three 10kDa, 13kDa and 16kDa prolamin promoters restrict the expression in the aleurone, subaleurone and the outer endosperm, similar to that of glutelin B gene promoters. As for the 26kDa globulin gene promoter and embryo globulin gene promoter, the prior directs expression in inner starchy region of endosperm and the activity of the latter is restricted in the aleurone and the embryo (Wu *et. al.,* 1998a; Qu

and Takaiwa, 2004 and Qu *et. al.*, 2008).

Among the four major rice seed storage proteins, glutelins are the most abundant in mature seeds accounting for about 80% of the total protein. There are 10 members in the glutelin gene family, divided into glutelin A and B sub-families (Takaiwa *et. al.*, 1996). Since glutelins are so diversified and abundant in rice, their promoters are the target for most rice seed-specific expression studies and a number of common *cis*-acting regulatory elements have been identified and characterized.

2.8.1.1 Endosperm-specific *cis*-acting regulatory elements

Study on comparing sequences of different glutelin genes promoters has identified several common consensus sequences. They are the AACA motif (AACAAACTCTATC), GCN4 motif (TGAGTCA), GCAA motif (GCAAAATGA), ACGT motif (GTACGTGG), endosperm box (TGTAAAG) and the prolamin-box (TGC/TAAAG). Extensive studies have been focused on the action of GCN4, prolamin-box and AACA motifs which are found in the 197bp GluB-1 minimal promoter conferring seed-specific expression in rice (Takaiwa *et. al.*, 1996).

GCN4 motif (GCAAAATGA) is conserved in promoters of many seed storage proteins found in rice, maize, barley and wheat. It locates at similar sites in promoters of all rice glutelin genes. It is also found to be an essential *cis*-acting element for rice seed-specific expression as a deletion mutation can dramatically reduces expression

level (Wu *et. al.*, 1998b). The GCN4 motif can be recognized and bound by basic domain/leucine zipper proteins (bZIP) such as Opaque-2 in maize, TGA1a in tobacco (de Pater *et. al.*, 1994) and SPA in wheat (Takaiwa *et. al.*, unpublished data). In rice, a bZIP, RISBZ1, has been identified as the functional transcription activator of GCN4 motif which has the same binding site with Opaque-2 on the GCN4 sequence. There are five RISBZ proteins in rice. Although all of them show ability to bind GCN4 motif, only binding with RISBZ1 can direct a high levels of transcriptional activation. The RISBZ1 expression is restricted in the aleurone and subaleurone layers and is suggested to form homodimer and heterodimer with other RISBZ proteins for its transcription activation activity (Onodera *et. al.*, 2001).

Prolamin-box (TG(C/T)AAAG) is found in promoters of all zein genes of maize and glutelin genes of rice. It is usually found to be separated by a few nucleotides with the GCN4 motif in the prolamin promoters; thus, it is suggested that the two motifs couple with each other and acts as a bifactorial endosperm box (Onodera *et. al.*, 2001). In the rice 197bp GluB-1 minimal promoter, mutation in the prolamin-box led to a reduction in seed-specific expression of GUS by 10-fold (Wu *et. al.*, 2000), suggesting its importance in maximizing transcriptional activity. The prolamin-box is found to be recognized by the Dof class PBF protein. In maize, PBF is found to be expressed in endosperm tissue and recognized specifically with the AAAG motif in the prolamin-box.

Mutation in the AAAG motif would abolish the binding activity. Since PBF shows protein-protein interaction with Opaque-2 in maize, it is suggested that PBF and Opaque-2 interacts with each other to confer transcriptional activity on maize zein genes (Vicente-Carbajosa *et. al.,* 1997).

Finally, AACA motif (AACAAACTCTATC) is a conserved sequence found in rice glutelin gene promoters and is also closely associated with the GCN4 motif (Wu *et. al.,* 2000). It is suggested that AACA motif is a negative regulator in non-seed tissues, but works together with the GCN4 motif in combination to direct endosperm-specific expression (Takaiwa *et. al.,* 1996). Similar with the action of prolamin-box, mutation in the AACA motif in the rice 197bp GluB-1 minimal promoter resulted in a complete loss of promoter activity and no GUS activity can be observed (Wu *et. al.,* 2000). A rice MYB protein, OSMYB5 has been identified as a recognition factor for the AACA motif. The AACA sequence is the target site of OSMYB5 as demonstrated by a binding assay (Suzuki *et. al.,* 1998).

2.8.1.2 Requirements to confer endosperm-specific expression in rice

In order to sort out the minimal requirement of *cis*-acting elements to confer rice seed-specific expression, Wu *et. al.* (1998b) focused on the promoter activity of the GluB-1 gene and successfully determined the shortest 197bp promoter fragment required to direct GUS expression in rice seed endosperm. The minimal 197bp promoter

fragment contained a TATA box, a ACGT motif, a AACA motif, a prolamin-box, and a GCN4 motif. Deletion and substitution have been done to mutate each *cis*-acting element to determine its role in directing seed-specific activity. Substitution mutation of the prolamin-box and ACGT motif led to a reduction in promoter activity by 10-fold and 4-fold respectively whereas, deletion of the GCN4 and AACA motifs would lead to a complete loss of promoter activity in rice seed.

Promoter activity has also been tested with these *cis*-acting elements existed as a trimeric form linked with a *CaMV35S* minimal promoter. Trimeric prolamin-box, AAGT motif and AACA motif failed to direct any seed-specific expression of GUS; whereas, only the trimeric GCN4 motif can confer seed-specific expression.

These results suggested that GCN4 is the only essential element for endosperm-specific expression in rice while the AACA motif, AAGT motif and prolamin-box are required for maximizing the expression level quantitatively. Therefore, a core GCN4 motif in combination with two seed-specific elements, either AACA motif, AAGT motif or the prolamin-box, is the minimal requirement conferring endosperm-specific expression in rice (Takaiwa *et. al.,* 1996; Wu *et. al.,* 1998b and Wu *et. al.,* 2000).

2.9 Project overview and hypothesis

Gene duplication of phytoene synthase and other carotenogenic enzymes is a common phenomenon in higher plants' genome. Multiple copies of phytoene synthase in tomato and maize enable the establishment of a tissue-specific carotenoid biosynthetic pathway in different tissues, for instance, the Sl*psy1* in chromoplast of tomato's fruit, the Sl*psy2* in green tissues, and the Zm*psy1* in amyloplast of maize's endosperm. The separation of tissue-specific carotenogensis pathway can help manipulating carotenoids content in a specific tissue without altering that in other tissues. Though the differential expression patterns of the three *Ospsy* have been revealed by real time PCR, it cannot determine the tissue-specificity of the activity of each *Ospsy*. Since the N-termini of the three *Ospsy* show the greatest variation, we hypothesize that (1) the three Os*PSY*s can be targeted to different plastids and show different preference for membrane association.

On the other hand, as it has been demonstrated that the expression of *Ospsy1* can direct enhanced carotenoid content in maize calli (Paine *et. al.*, 2005), it is a possible candidate for making Golden Rice. We hypothesize that (2) a new version of Golden Rice can be developed by introducing *Ospsy1*, rice PDS and ZDS activities back into rice endosperm, so that the use of bacterial CrtI and mixing of genes from different plant origins can be avoided to gain public acceptance.

It has been demonstrated that expression of *Ospsy1* was absent in rice endosperm. One of the possible reasons for such phenomenon is the lack of sufficient seed-specific elements available in the *Ospsy1* promoter. We hypothesize that (3) the introduction of seed-specific elements, such as GCN4 motif, can introduce the seed expression activity of the *Ospsy1* promoter.

In order to test the three hypotheses mentioned above, in this project, we propose to:

(1) Determine the tissue-specificity of the action of *Ospsy1* and *Ospsy2* as reflected by changes in carotenoid content under constitutive expression of the two *Ospsys*

(2) Test the possibility of using *Ospsy1* and *Ospsy2* as the candidate to produce Golden Rice under endosperm-specific expression of the two *Ospsys*

(3) Introduce the seed expression activity of the *Ospsy1* promoter by introduction addition of rice endosperm-specific *cis*-acting element, GCN4 motif

Chapter 3: Materials and Methods

3.1 Chemicals

All the chemicals used were of reagent grade or molecular grade, and were purchased from Sigma-Aldrich Corp. or Bio-Rad Laboratories, Inc. unless otherwise specified. Restriction enzymes and other enzymes for molecular biology experiments were obtained from New England Biolabs, Inc. and Promega Corp. All the solvents and standards used in UPLC analysis were of HPLC grade and were obtained from Sigma-Aldrich Corp., Wako Pure Chemical Industries, Ltd., CaroteNature and Merck & Co., Inc.

3.2 Vectors and bacterial strains in regular cloning

The pGEM-T Vector system was purchased from Promega Corp for gene cloning. The pSB130, pSB130/GUS and pBI121 vectors were provided by Prof. Sun, S.S.M. (The Chinese University of Hong Kong, Hong Kong). The pSB130 and pSB130/GUS super binary vectors were used for rice transformation; while the pBI121 vector was used as the source of *CaMV35S* promoter and *CaMV35S* minimal promoter. *E. coli* DH5α was used as a host of pGEM-T, pSB130, pSB130/GUS and pBI121 vectors manipulation and maintenance. *E. coli* BL21 and Rosetta (2), kindly provided by Prof. Wong, K.B. (The Chinese University of Hong Kong, Hong Kong), were used together

with the pET system for protein expression. *Agrobacterium tumefaciens* strain EHA105 was used in rice transformation.

3.3 Plant materials

All *Oryza sativa* subsp. *Japonica* seeds used in this project, both cultivar 9983 and WYJ3, were kindly provided by Dr. Liu, Q.Q. (Agricultural College of Yangzhou University, China).

3.4 Construction of gene cassettes for plant transformation

3.4.1 Construction of gene cassettes for *Ospsy1* and *Ospsy2* study

The vector pSB130 used in rice transformation was a 9800bp super binary vector with two separate T-DNA regions. This vector contains two sets of left and right borders, therefore is ready to transfer two sets of T-DNA into the rice's genome. One of the T-DNA set contains a hygromycin resistance gene, followed by a NOS terminator, under the control of *CaMV35S* promoter while the other T-DNA set contains multiple cloning sites and a NOS terminator. This vector can be selected with kanamycin.

In the course of making the four gene cassettes for *Ospsy1* and *Ospsy2* study, the following primers were used as shown in Table 3.1.

Table 3.1 Primer list for *Ospsy1* and *Ospsy2* gene cassettes construction

Name	Sequence	Length
A01	KpnI 5'-GCGCGCGGTACCATGGCGGCCATCACGCTCCTACG-3'	(35mer)
A02	EcoRI 5'-AGAATTCCTACTTCTGGCTATTTCTC-3'	(26mer)
A03	KpnI 5'-GCGCGCGGTACCATGGCGTCCTCCTCGTCGGCGGCG-3'	(36mer)
A04	EcoRI 5'-AGAATTCTCATGATGCAACTGCCGCTC-3'	(27mer)
A05	PstI 5'-GCATCGGCTGCAGCCCACAGATGGTTAGAGAGGCTTAC-3'	(38mer)
A06	KpnI 5'-CGCGCGGGTACCCGTGTTCTCTCCAAATGAAATGAACTT-3'	(39mer)
A07	PstI 5'-GCATCGGCTGCAGAAGCTTCACCCTCAATATTTGGAAACA-3'	(40mer)
A08	KpnI 5'-CGCGCGGGTACCGGATCCGTTGTTGTAGGACTAATGAAC-3'	(39mer)

3.4.1.1 Cloning of *Ospsy1* and *Ospsy2* from rice

Ospsy1 cDNA was cloned by RT-PCR using total RNA extracted from wild type rice *Japonica* cultivar 9983 leaves while the cDNA of *Ospsy2* was acquired from Rice Genome Resource Centre (RGRC).

3.4.1.1.1 Total RNA extraction

Two grams of leaves from wild type rice *Japonica* cultivar 9983 were collected and ground into powder in liquid nitrogen. Five mililitre equilibrated phenol (Ultrapure, MB grade, pH8.0) and equal volume of extraction buffer (50mM Tris, 20mM NaCl, 2mM EDTA, 1% SDS, pH 9.0) were added to the leaf powder. The mixture was mixed by inverting and then kept in ice for 10 minutes. The homogenate was centrifuged at 1,485 rcf at 4 °C for 20 minutes and the supernatant was collected. Two volumes of cold 100% ethanol were added to the supernatant and the mixture was kept in ice for 30 minutes for precipitation after mixing. The mixture was centrifuged again at 1,485 rcf at 4 °C for 20 minutes. The pellet obtained was washed with cold 70% ethanol, followed by centrifugation at 1,485 rcf at 4 °C for 20 minutes. The pellet was dried by removing excess 70% ethanol and was resuspended in 1ml cold 2M LiCl before keeping at 4 °C overnight for RNA precipitation. The extract was centrifuged at 25,000 rcf at 4 °C for 20 minutes. The supernatant was discarded and the pellet was resuspended in 1ml cold 70% ethanol. This centrifugation and resuspension steps were done twice to remove

impurities. The pellet was air dried at room condition and was resuspended in 50µl cold DEPC-treated water.

3.4.1.1.2 Amplification of cDNA of Ospsy1 by RT-PCR

RT-PCR was performed using 2µg leaves total RNA (Section 3.4.1.1.1). Reverse transcription was done using gene specific reverse primer A02 (Table 3.1) and SuperScript™ II reverse transcriptase (Invitrogen).

RNA (2µg) was mixed with 1µl 25mM reverse primer A02 and 8µl DEPC treated water. The mixture was incubated at 70°C for 10 minutes and then kept on ice for 1 minute. The reverse transcription was done in 1X First Strand Buffer, 10mM DTT, 1mM dNTPs and 1 unit of SuperScript™ II reverse transcriptase (Invitrogen) at 42 °C for 90 minutes. The reaction was halted by keeping the reaction mixture at 75 °C for 15 minutes.

The first strand product was used as the template for amplifying the *Ospsy1* cDNA by PCR. A 20µl PCR reaction mixture containing 2µl first strand product, 1X High Fidelity PCR buffer, 0.2mM dNTPs, 2mM $MgSO_4$, 0.5µM forward primer A01, 0.5µM reverse primer A02 (Table 3.1) and 1 unit of Platinum® *Taq* High Fidelity polymerase (Invitrogen) was prepared. The PCR conditions were as follow: 94°C for 10 minutes, then 30 cycles at 94°C for 1 minute, 50 °C for 1 minute and 72°C for 3 minutes, followed by 1 cycle at 72°C for 7 minutes. The reaction was maintained at 15°C after

cycling. A 5' KpnI and a 3' EcoRI restriction site were introduced into the *Ospsy1* cDNA through PCR amplification mentioned above. The amplified fragment was ligated into pGEM®-T vector for later manipulation. Sequence fidelity of the amplified fragment was confirmed by DNA sequencing (Section 3.4.3), and was shown to be identical with the 1236bp cDNA of *Ospsy1* (GenBank, accession number: AJ715786) as shown in Figure 3.1.

```
   1 ATGGCGGCCA TCACGCTCCT ACGTTCAGCG TCTCTTCCGG GCCTCTCCGA CGCCCTCGCC
  61 CGGGACGCTG CTGCCGTCCA ACATGTCTGC TCCTCCTACC TGCCCAACAA CAAGGAGAAG
 121 AAGAGGAGGT GGATCCTCTG CTCGCTCAAG TACGCCTGCC TTGGCGTCGA CCCTGCCCCG
 181 GGCGAGATTG CCCGGACCTC GCCGGTGTAC TCCAGCCTCA CCGTCACCCC TGCTGGAGAG
 241 GCCGTCATCT CCTCGGAGCA GAAGGTGTAC GACGTCGTCC TCAAGCAGGC AGCATTGCTC
 301 AAACGCCACC TGCGCCCACA ACCACACACC ATTCCCATCG TTCCCAAGGA CCTGGACCTG
 361 CCAAGAAACG GCCTCAAGCA GGCCTATCAT CGCTGCGGAG AGATCTGCGA GGAGTATGCC
 421 AAGACCTTTT ACCTTGGAAC TATGCTCATG ACGGAGGACC GACGGCGCGC CATATGGGCC
 481 ATCTATGTGT GGTGTAGGAG GACAGATGAG CTTGTAGATG GACCAAATGC CTCGCACATC
 541 ACACCGTCAG CCCTGGACCG GTGGGAGAAG AGGCTTGATG ATCTCTTCAC CGGACGCCCC
 601 TACGACATGC TTGATGCTGC ACTTTCTGAT ACCATCTCCA AGTTTCCTAT AGATATTCAG
 661 CCTTTCAGGG ACATGATAGA AGGGATGCGG TCAGACCTCA GAAAGACTAG ATACAAGAAC
 721 TTCGACGAGC TCTACATGTA CTGCTACTAT GTTGCTGGAA CTGTGGGGCT AATGAGTGTT
 781 CCTGTGATGG GTATTGCACC CGAGTCGAAG GCAACAACTG AAAGTGTGTA CAGTGCTGCT
 841 TTGGCTCTCG GCATTGCAAA CCAGCTCACA AATATACTCC GTGACGTTGG AGAGGACGCG
 901 AGAAGAGGGA GGATATATTT ACCACAAGAT GAACTTGCAG AGGCAGGGCT CTCTGATGAG
 961 GACATCTTCA ATGGCGTTGT GACTAACAAA TGGAGAAGCT TCATGAAGAG ACAGATCAAG
1021 AGAGCTAGGA TGTTTTTTGA GGAGGCAGAG AGAGGGGTGA CCGAGCTCAG CCAGGCAAGC
1081 CGGTGGCCGG TCTGGGCGTC TCTGTTGTTA TACCGGCAAA TCCTTGACGA GATAGAAGCA
1141 AACGATTACA ACAACTTCAC AAAGAGGGCG TACGTTGGGA AGGCGAAGAA ATTGCTAGCG
1201 CTTCCAGTTG CATATGGTAG ATCATTGCTG ATGCCCTACT CACTGAGAAA TAGCCAGAAG
1261 TAG
```

MAAITLLRSASLPGLSDALARDAAAVQHVCSSYLPNNKEKKRRWILCSLKYACLGVD
PAPGEIARTSPVYSSLTVTPAGEAVISSEQKVYDVVLKQAALLKRHLRPQPHTIPIVPKD
LDLPRNGLKQAYHRCGEICEEYAKTFYLGTMLMTEDRRRAIWAIYVWCRRTDELVDG
PNASHITPSALDRWEKRLDDLFTGRPYDMLDAALSDTISKFPIDIQPFRDMIEGMRSDL
RKTRYKNFDELYMYCYYVAGTVGLMSVPVMGIAPESKATTESVYSAALALGIANQLT
NILRDVGEDARRGRIYLPQDELAEAGLSDEDIFNGVVTNKWRSFMKRQIKRARMFFE
EAERGVTELSQASRWPVWASLLLYRQILDEIEANDYNNFTKRAYVGKAKKLLALPVA
YGRSLLMPYSLRNSQK (420 amino acids)

Figure 3.1 Full length cDNA and amino acids sequence of 1263bp rice *Ospsy1* (AJ715786)

3.4.1.1.3 Amplification of cDNA of Ospsy2 by PCR

Ospsy2 cDNA (Clone ID: J033025L21) was acquired from Rice Genome Resource Centre (RGRC). A 5' KpnI and a 3' EcoRI restriction sites were introduced to flank the 1197bp *Ospsy2* cDNA through PCR amplification. A 20µl PCR reaction mixture containing 1ng purchased plasmid, 1X High Fidelity PCR buffer, 0.2mM dNTPs, 1mM $MgSO_4$, 0.5µM forward primer A03, 0.5µM reverse primer A04 (Table 3.1) and 1 unit of Platinum® *Taq* High Fidelity polymerase (Invitrogen) was prepared. The PCR conditions were as follow: 94°C for 10 minutes, then 30 cycles at 94°C for 1 minute, 50 °C for 1 minute and 72°C for 3 minutes, followed by 1 cycle at 72°C for 7 minutes. The reaction was maintained at 15°C after cycling. The amplified fragment was then ligated into pGEM®-T vector and the sequence fidelity of the amplified fragment was confirmed by DNA sequencing (Section 3.4.3), which was shown to be identical with the 1197bp cDNA of *Ospsy2* (GenBank, accession number: AK073290) as shown in Figure 3.2.

```
   1 ATGGCGTCCT CCTCGTCGGC GGCGGCGCTC TGGACGGCGG CGCCCCACCC CCACGGCAGC
  61 TGCATCAGGA TCCACGCCAT CTTCCACCAG CGTCACCAGC GGAGGGGGAG GAGGCCGGTC
 121 GTCGTCGCCT CGTCGGTGAG GCCGCTGCAG GCCGCGAGCC TGGCGGTGGC CACGGCGCCG
 181 GTGGCCGTGG CGTCGAGGAG GACGGCGGCG GAGGAGGCCG TCTACGAGGT CGTGCTGCGG
 241 CAGGCGGCGC TGGTGGAGGA GGCCACCCAC CGCCGCGGCG CGGGGGCGCC GCGGTGGGCG
 301 GAGGAGGACG CCGTCGACTG GGGCCTCCTC CTCGGCGACG CCTACCACCG CTGCGGCGAG
 361 GTCTGCGCCG AGTACGCCAA GACCTTCTAC CTAGGTACTC AGCTTATGAC TCCTGAAAGG
 421 CGCAAAGCTG TCTGGGCAAT CTATGTATGG TGCAGAAGAA CTGATGAACT GGTAGATGGC
 481 CCTAACTCGT CTTACATTAC ACCAAAGGCA CTTGATCGAT GGGAGAAGAG ATTAGAAGAT
 541 CTCTTCGAAG GCAGGCCATA TGATATGTAT GATGCAGCCC TCTCGGACAC AGTGTCAAAG
 601 TTTCCAGTAG ATATCCAGCC ATTCAAAGAC ATGATTGAAG GAATGAGGCT TGACCTGTGG
 661 AAATCAAGGT ATAGGAGCTT TGATGAGCTC TACCTCTACT GCTACTACGT TGCTGGCACG
 721 GTTGGTCTCA TGACAGTACC GGTGATGGGG ATTGCCCCCG ACTCGAAGGC CTCAACCGAG
 781 AGCGTGTACA ACGCTGCGCT AGCTCTTGGG ATCGCCAACC AGCTGACGAA TATTCTCAGA
 841 GACGTAGGCG AAGACTCAAG GAGGGGAAGA ATCTACCTTC CATTGGATGA ATTGGCAGAG
 901 GCAGGTCTGA CAGAAGAAGA CATATTCAGA GGGAAAGTGA CTGATAAATG GAGGAAGTTC
 961 ATGAAGGGAC AAATTCTGCG TGCCAGGTTA TTCTTTGATG AGGCGGAGAA GGGCGTTGCG
1021 CATCTAGACT CTGCGAGTAG ATGGCCGGTT CTGGCATCTT TGTGGTTATA CCGGCAGATC
1081 CTTGATGCTA TCGAAGCAAA CGACTACAAC AACTTCACCA AGCGCGCGTA TGTAAACAAG
1141 GCAAAGAAGC TGCTGTCTTT ACCGGTCGCT TATGCAAGAG CGGCAGTTGC ATCATGA
```

MASSSSAAALWTAAPHPHGSCIRIHAIFHQRHQRRGRRPVVVASSVRPLQAASLAVA
TAPVAVASRRTAAEEAVYEVVLRQAALVEEATHRRGAGAPRWAEEDAVDWGLLLG
DAYHRCGEVCAEYAKTFYLGTQLMTPERRKAVWAIYVWCRRTDELVDGPNSSYITP
KALDRWEKRLEDLFEGRPYDMYDAALSDTVSKFPVDIQPFKDMIEGMRLDLWKSRY
RSFDELYLYCYYVAGTVGLMTVPVMGIAPDSKASTESVYNAALALGIANQLTNILRD
VGEDSRRGRIYLPLDELAEAGLTEEDIFRGKVTDKWRKFMKGQILRARLFFDEAEKG
VAHLDSASRWPVLASLWLYRQILDAIEANDYNNFTKRAYVNKAKKLLSLPVAYARA
AVAS (398 amino acids)

Figure 3.2 Full length cDNA and amino acids sequence of 1197bp rice *Ospsy2* (AK073290)

3.4.1.2 Cloning of constitutive *CaMV35S* promoter

3.4.1.2.1 Preparation of pBI121 vector

CaMV35S promoter was cloned from pBI121 vector. In order to obtain adequate amount of plasmid, pBI121 was used to transform *E.coli* DH5α using heat-shock method.

pBI121 (5ng) was added into 160μl DH5α competent cells. The mixture was kept on ice for 30 minutes, and then incubated at 37°C for 5 minutes. LB broth (750μl) was added into the culture for recovery, and was incubated at 37 °C with shaking at 500 rpm for an hour. The bacterial cells were pelleted by centrifugation at 300 rpm and were resuspended in 100μl LB broth. All the bacterial culture was spread onto a LB plate with 50μg/ml kanamycin. The plate was incubated at 37 °C for 16 hours. Single colony was picked and inoculated into 3ml of LB broth with the same antibiotic composition as the LB plate. Two hundred microlitres of the transformed cells were kept as stock with 50% glycerol and stored at - 80 °C, the rest of them were used for plasmid purification using the Wizard® *Plus* Minipreps DNA Purification System (Promega).

3.4.1.2.2 Amplification of CaMV35S promoter by PCR

The 801bp *CaMV35S* promoter was cloned by PCR using pBI121 vector as the DNA template. A 5' PstI and a 3' KpnI restriction sites were introduced to flank the *CaMV35S* promoter through PCR amplification. A 20μl PCR reaction mixture

containing 1μg pBI121, 1X GoTaq® reaction buffer, 0.2mM dNTPs, 3mM $MgCl_2$, 0.5μM forward primer A05, 0.5μM reverse primer A06 (Table 3.1) and 1 unit of GoTaq® DNA polymerase (Promega) was prepared. The PCR conditions were as follow: 94°C for 10 minutes, then 30 cycles at 94°C for 1 minute, 50 °C for 1 minute and 72°C for 90 seconds, followed by 1 cycle at 72°C for 7 minutes. The reaction was maintained at 15°C after cycling. The amplified fragment was ligated into pGEM®-T vector and the sequence fidelity of the amplified fragment was confirmed by DNA sequencing (Section 3.4.3) as shown in Figure 3.3.

```
  1 CCCACAGATG GTTAGAGAGG CTTACGCAGC AGGTCTCATC AAGACGATCT ACCCGAGCAA
 61 TAATCTCCAG GAAATCAAAT ACCTTCCCAA GAAGGTTAAA GATGCAGTCA AAAGATTCAG
121 GACTAACTGC ATCAAGAACA CAGAGAAAGA TATATTTCTC AAGATCAGAA GTACTATTCC
181 AGTATGGACG ATTCAAGGCT TGCTTCACAA ACCAAGGCAA GTAATAGAGA TTGGAGTCTC
241 TAAAAAGGTA GTTCCCACTG AATCAAAGGC CATGGAGTCA AAGATTCAAA TAGAGGACCT
301 AACAGAACTC GCCGTAAAGA CTGGCGAACA GTTCATACAG AGTCTCTTAC GACTCAATGA
361 CAAGAAGAAA ATCTTCGTCA ACATGGTGGA GCACGACACA CTTGTCTACT CCAAAAATAT
421 CAAAGATACA GTCTCAGAAG ACCAAAGGGC AATTGAGACT TTTCAACAAA GGGTAATATC
481 CGGAAACCTC CTCGGATTCC ATTGCCCAGC TATCTGTCAC TTTATTGTGA AGATAGTGGA
541 AAAGGAAGGT GGCTCCTACA AATGCCATCA TTGCGATAAA GGAAAGGCCA TCGTTGAAGA
601 TGCCTCTGCC GACAGTGGTC CCAAAGATGG ACCCCCACCC ACGAGGAGCA TCGTGGAAAA
661 AGAAGACGTT CCAACCACGT CTTCAAAGCA AGTGGATTGA TGTGATATCT CCACTGACGT
721 AAGGGATGAC GCACAATCCC ACTATCCTTC GCAAGACCCT TCCTCTATAT AAGGAAGTTC
781 ATTTCATTTG GAGAGAACAC G
```

Figure 3.3 Full length sequence of 801bp *CaMV35S* promoter

3.4.1.3 Cloning of endosperm-specific rice glutelin-1 (Gt-1) promoter

Rice glutelin-1 promoter was cloned by PCR using genomic DNA extracted from wild type rice *Japonica* cultivar 9983 leaves using the CTAB method.

3.4.1.3.1 Genomic DNA extraction

Similar to the total RNA extraction, 2 grams of wild type rice *Japonica* cultivar 9983 leaves were collected and ground into powder in liquid nitrogen. CTAB buffer (500µl) (100mM Tris-HCL, 1.4M NaCl, 20mM EDTA, 2% CTAB, 0.2% 2-mercaptoethanol, pH 8.0) was added to the powder. The homogenate was incubated at 60 °C with shaking at 750 rpm for 40 minutes. After that, 500µl chloroform:isoamyl alcohol (24:1, v/v) was mixed with the homogenate before centrifugation at 11,485 rcf at 4 °C for 15 minutes. The supernatant was collected and mixed with 330µl cold isopropanol by inversion before being kept at -20 °C for 2 hours. After precipitation, the mixture was centrifuged at 18,188 rcf at 4 °C for 20 minutes. The supernatant was discarded and the pellet was washed with 300 µl cold 70% ethanol. This spinning and washing steps were done twice to remove impurities. The genomic DNA pellet was air dried and resuspended into 50µl of cold sterilized water.

3.4.1.3.2 Amplification of Gt-1 promoter by PCR

The 1861bp Gt-1 promoter was cloned by PCR using genomic DNA extracted (Section 3.4.1.3.1) as the DNA template. A 5' PstI and a 3' KpnI restriction sites were

introduced to flank the promoter through PCR amplification. A 20μl PCR reaction mixture containing 2μg genomic DNA, 1X High Fidelity PCR buffer, 0.2mM dNTPs, 2mM $MgSO_4$, 0.5μM forward primer A07, 0.5μM reverse primer A08 (Table 3.1) and 1 unit of Platinum® *Taq* High Fidelity polymerase (Invitrogen) was prepared. The PCR conditions were as follow: 94°C for 10 minutes, then 30 cycles at 94°C for 1 minute, 50 °C for 1 minute and 72°C for 3 minutes, followed by 1 cycle at 72°C for 7 minutes. The reaction was maintained at 15°C after cycling. The amplified fragment was ligated into pGEM®-T vector, and the sequence fidelity of the amplified fragment was confirmed by DNA sequencing (Section 3.4.3) as shown in Figure 3.4.

```
   1 AAGCTTCACC CTCAATATTT GGAAACATTT ATCTAGGTTG TTTGTGTCCA GGCCTATAAA
  61 TCATACATGA TGTTGTCGTA TTGGATGTGA ATGTGGTGGC GTGTTCAGTG CCTTGGATTT
 121 GAGTTTGATG AGAGTTGCTT CTGGGTCACC ACTCACCATT ATCGATGCTC CTCTTCAGCA
 181 TAAGGTAAAA GTCTTCCCTG TTTACGTTAT TTTACCCACT ATGGTTGCTT GGGTTGGTTT
 241 TTTCCTGATT GCTTATGCCA TGGAAAGTCA TTTGATATGT TGAACTTGAA TTAACTGTAG
 301 AATTGTATAC ATGTTCCATT TGTGTTGTAC TTCCTTCTTT TCTATTAGTA GCCTCAGATG
 361 AGTGTGAAAA AAACAGATTA TATAACTTGC CCTATAAATC ATTTGAAAAA AATATTGTAC
 421 AGTGAGAAAT TGATATATAG TGAATTTTTA AGAGCATGTT TTCCTAAAGA AGTATATATT
 481 TTCTATGTAC AAAGCCATTG AAGTAATTGT AGATACAGGT AATTAGACTT TTTGGACTTA
 541 CACTGCTACC TTTAAGTAAC AATCATGAGC AATAGTGTTG CAATGATATT TAGGCTGCAT
 601 TCGTTTACTC TCTTGATTTC CATGAGCACG CTTCCCAAAC TGTTAAACTC TGTGTTTTTT
 661 GCCAAAAAAA AATGTATAGG AAAGTTGCTT TTAAAAAATC ATATCAATCC ATTTTTTAAG
 721 TTATAGCTAA TACTTAATTA ATCATGCGCT AATAAGTCAC TCTGTTTTTC GTACTAGAGA
 781 GATTGTTTTG AACCAGCACT CAAGAACACA GCCTTAACCC AGCCAAATAA TGCTACAACC
 841 TACCAGTCCA CACCTCTTGT AAAGCATTTG TTGCATGGAA AAGCTAAGAT GACAGCAACC
 901 TGTTCAGGAA AACACTGACA AGGTCATAGG GAGAGGGAGC TTTTGGAAAG GTGCCGTGCA
 961 GTTCAAACAA TTAGTTAGCA GTAGGGTGTT GGTTTTTGCT CACAGCAATA AGAAGTTAAT
1021 CATGGTGTAG GCAACCCAAA TAAAACACCA AAATATGCAC AAGGCAGTTT GTTGTATTCT
1081 GTAGTACAGA CAAAACTAAA AGTAATGAAA GAAGATGTGG TGTTAGAAAA GGAAACAATA
1141 TCATGAGTAA TGTGTGAGCA TTATGGGACC ACGAAATAAA AAGAACATTT TGATGAGTCG
1201 TGTATCCTCG ATGAGCCTCA AAAGTTCTCT CACCCCGGAT AAGAAACCCT TAAGCAATGT
1261 GCAAAGTTTG CATTCTCCAC TGACATAATG CAAAATAAGA TATCATCGAT GACATAGCAA
1321 CTCATGCATC ATATCATGCC TCTCTCAACC TATTCATTCC TACTCATCTA CATAAGTATC
1381 TTCAGCTAAA TGTTAGAACA TAAACCCATA AGTCACGTTT GATGAGTATT AGGCGTGACA
1441 CATGACAAAT CACAGACTCA AGCAAGATAA AGCAAAATGA TGTGTACATA AAACTCCAGA
1501 GCTATATGTC ATATTGCAAA AAGAGGAGAG CTTATAAGAC AAGGCATGAC TCACAAAAAT
1561 TCACTTGCCT TTCGTGTCAA AAAGAGGAGG GCTTTACATT ATCCATGTCA TATTGCAAAA
1621 GAAAGAGAGA AAGAACAACA CAATGCTGCG TCAATTATAC ATATCTGTAT GTCCATCATT
1681 ATTCATCCAC CTTTCGTGTA CCACACTTCA TATATCATAA GAGTCACTTC ACGTCTGGAC
1741 ATTAACAAAC TCTATCTTAA CATTTAGATG CAAGAGCCTT TATCTCACTA TAAATGCACG
1801 ATGATTTCTC ATTGTTTCTC ACAAAAAGCA TTCAGTTCAT TAGTCCTACA ACAACGGATC
1861 C
```

Figure 3.4 Full length sequence of 1861bp rice Gt-1 promoter

3.4.1.4 Construction of gene cassettes for *Ospsy1* and *Ospsy2* driven by *CaMV35S* and rice Gt-1 promoter

In order to transfer the gene cassettes into the rice's genome, the *CaMV35S* promoter, the Gt-1 promoter and the two *Ospsy* cDNA have to be cloned into the multiple cloning site of pSB130 vector.

To transfer the *CaMV35S* promoter into pSB130, a 5' PstI and a 3' KpnI restriction sites have been introduced into the *CaMV35S* promoter through PCR amplification as mentioned above, the promoter was excised by restriction digestion with PstI and KpnI, and ligated to pSB130, which had also been cut by the same enzymes forming the pSB130/$35S_{pro}$ vector as shown in Figure 3.5 (A).

After that, the two *Ospsy* cDNAs, *Ospsy1* and *Ospsy2*, were excised by restriction digestion with KpnI and EcoRI. The two fragments were then ligated to pSB130/$35S_{pro}$, which had been cut using the same enzymes giving pSB130/$35S_{pro}$ */Ospsy1* and pSB130/$35S_{pro}$/*Ospsy2* vectors. The same strategy was also employed in making the two Gt-1 promoter-driven constructs. The overall cloning strategy of making the four *Ospsy1* and *Ospsy2* constructs was shown in Figure 3.5.

(A) Transferring *CaMV35S* and Gt-1 promoters into pSB130

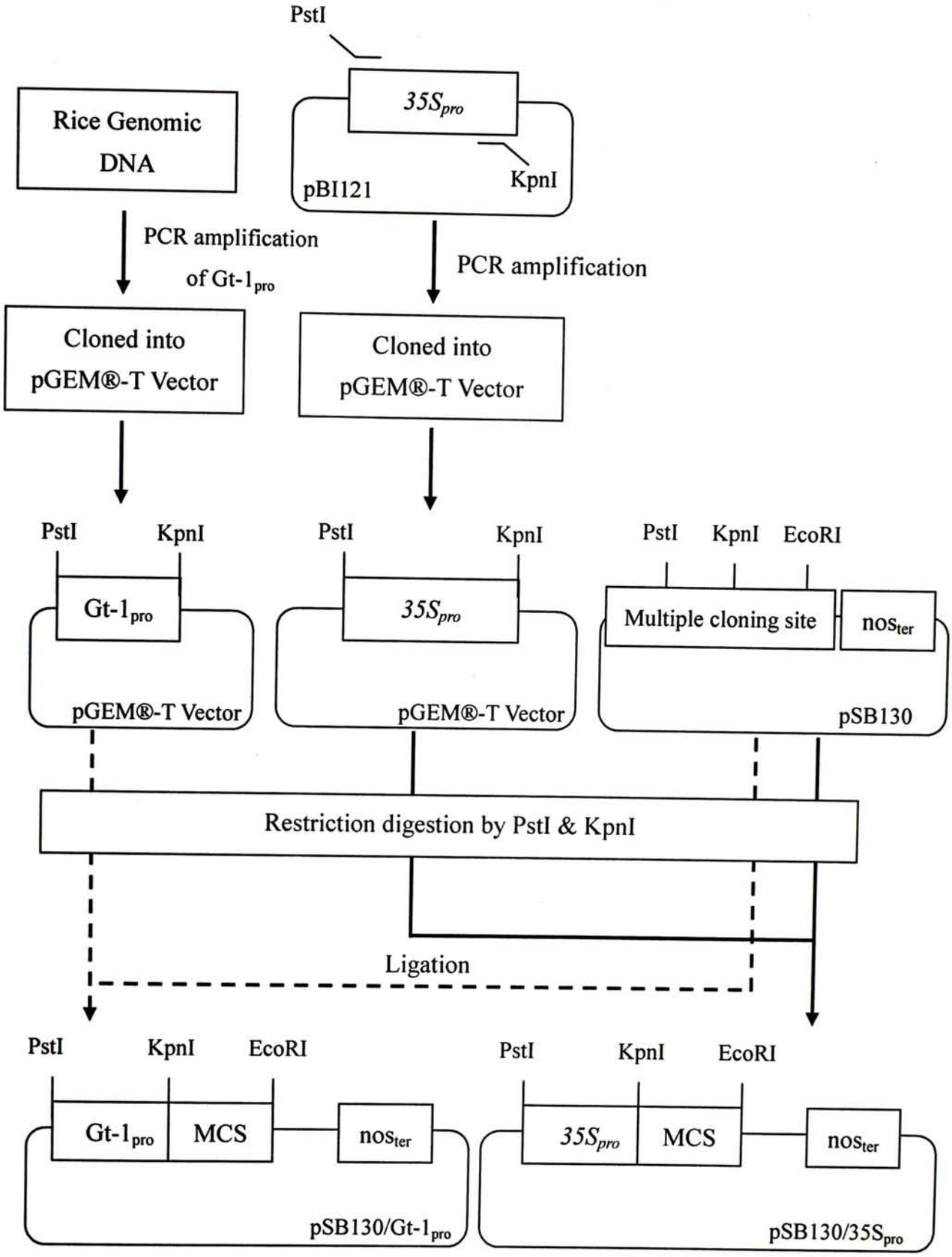


Figure 3.5 Strategy of construction of gene cassettes for *Ospsy1* and *Ospsy2* driven by *CaMV35S* and rice Gt-1 promoters

(A) Transferring *CaMV35S* and Gt-1 promoters into pSB130

(B) Transferring *Ospsy1* and *Ospsy2* into pSB130 with *CaMV35S* and Gt-1 promoter

(B) Transferring *Ospsy1* and *Ospsy2* into pSB130 with *CaMV35S* and Gt-1 promoters

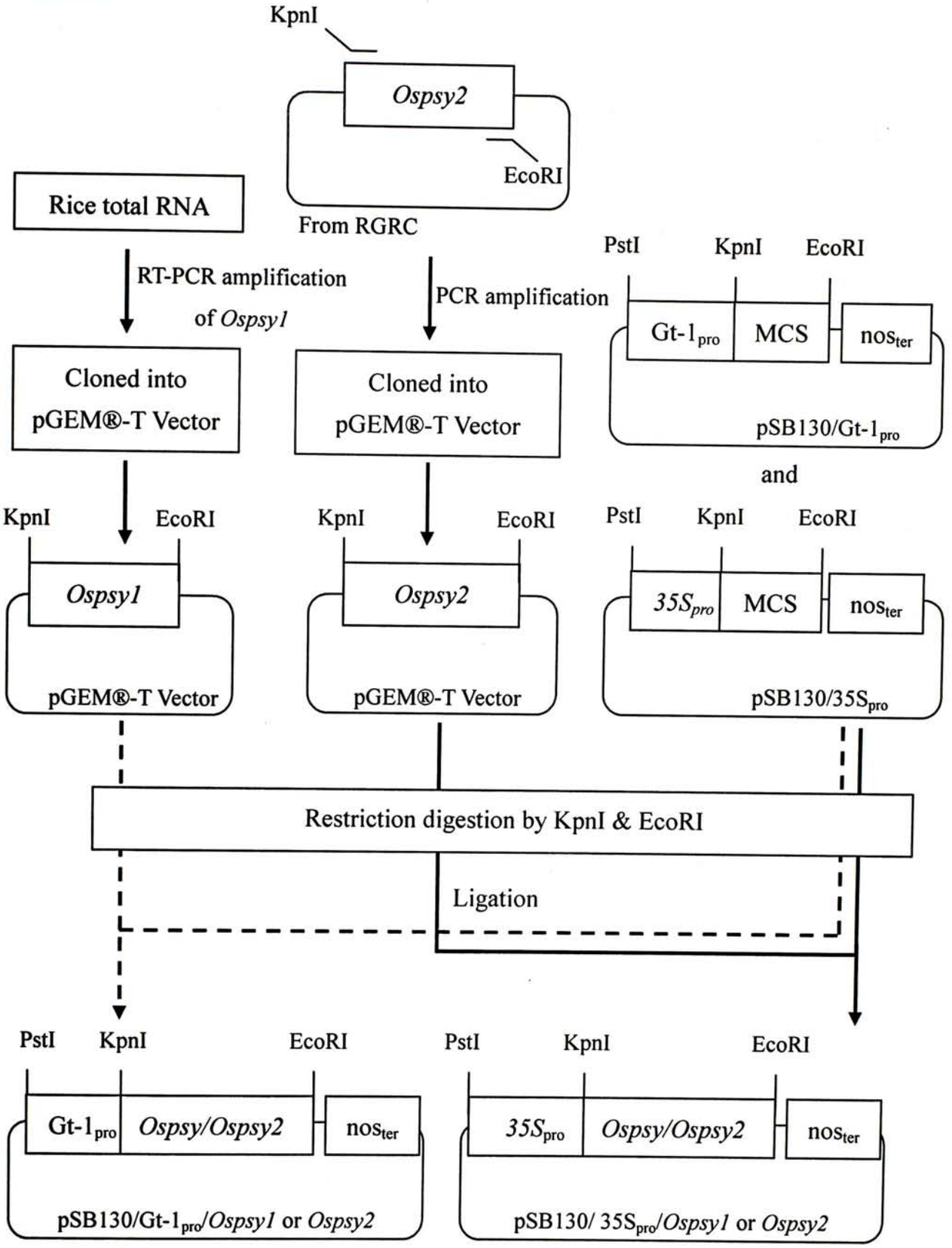


Figure 3.5 Strategy of construction of gene cassettes for *Ospsy1* and *Ospsy2* driven by *CaMV35S* and rice Gt-1 promoters

(A) Transferring *CaMV35S* and Gt-1 promoters into pSB130

(B) Transferring *Ospsy1* and *Ospsy2* into pSB130 with *CaMV35S* and Gt-1 promoters

3.4.2 Construction of gene cassettes for promoter analysis

The vector pSB130/GUS used in rice transformation is a 12900bp super binary vector with two separate T-DNA regions. This vector contains two sets of left and right borders, therefore is ready to transfer two sets of T-DNA into the rice's genome. One of the T-DNA contains a hygromycin resistance gene same as pSB130 while the other T-DNA contains multiple cloning sites, a GUS gene and a NOS terminator. This vector can also be selected with kanamycin.

In the course of making the five gene cassettes for modified *Ospsy1* promoter study, the following primers were used as shown in Table 3.2.

Table 3.2 Primer list for modified promoter gene cassettes construction

Name	Sequence	Length
B01	EcoRI 5'-GCGCGAATTCTCTCTCAACGATAAACTCAACAGTATG-3'	(37mer)
B02	KpnI 5'-CGCGCGGTACCCGTTGCTTTAATTTGGTGATAAACACA-3'	(38mer)
B03	HindIII 5'-GCGCGCAAGCTTACACTCACTTGGTCTGTTTCTTTTTAA-3'	(39mer)
B04	NcoI 5'-GCGCGCCCATGGTATTATACTGGATGGGATCAATCGC-3'	(37mer)
B05	HindIII 5'-GCGCGCAAGCTTGTGATATCTCCACTGACGTAAGGG-3'	(36mer)
B06	NcoI 5'-GCGCGCCCATGGCGTGTTCTCTCCAAATGAAATGAACTTC-3'	(40mer)
A07	PstI 5'-GCATCGGCTGCAGAAGCTTCACCCTCAATATTTGGAAACA-3'	(40mer)
A08	KpnI 5'-CGCGCGGGTACCGGATCCGTTGTTGTAGGACTAATGAAC-3'	(39mer)
B09	5'-GCGCGCGTTTGTCATGGCTGAGTCATG-3'	(27mer)
B10	5'-GCGCGCCATGACTCAGCCATGACAAAC-3'	(27mer)
B11	EcoRI PstI KpnI 5'-AATTCCTGCAGAGGCCTGGTACCC-3'	(24mer)
B12	NcoI KpnI PstI 5'-CATGGGGTACCAGGCCTCTGCAGG-3'	(24mer)

3.4.2.1 Cloning of full length and fragments of *Ospsy1* promoter

The sequence of the *Ospsy1* promoter was determined as the 1500bp genomic DNA sequence, as shown in Figure 3.6, upstream of the start codon (ATG) of the *Ospsy1* gene in the *Japonica* rice genome. The full length *Ospsy1* promoter (Fragments I + II) used in this project referred to this 1500bp sequence. Fragment I referred to the first 1369bp (Figure 3.7 A) from the 5' end of this 1.5kb sequence while Fragment II referred to the last 131bp (Figure 3.7 B) of the full sequence housing the TATA box of the *Ospsy1* promoter.

```
   1 TCTCTCAACG ATAAACTCAA CAGTATGTAA CTGAAGGGAT CAGTACTTGA GATGAAAAAG
  61 AAACAAGGAC AGAGTAGTAA AATCAGGCTA CCATCCATTT AGGGCATGTT TGGAGCAGAG
 121 TATAAAACAT GTGAATAACA CAGGAAACAA AACTTAGGAA TTTGTTTGTA TTGAAAAACA
 181 CAGCAAATTA ATGTGTATGA ACAAAAGTAA TAACAAAACA CCTGAATCAT TTTTCTCGTA
 241 TCATGTCCCT TGTGATCTTT ATTACTTCTA CTTCGATTCC ACTGGTATAA ACAGATTTTC
 301 GTTTTTGACA TTGGTATGGT ATTCAGATAG AATACAGCTT CAATAATTAA TTTATATTGT
 361 ACTAATATGT TTGTAAAGAA CTTGGTCCAA AAAAAATGTG TTTGTAAAAA CATAACGAAC
 421 ATATAATACT TTTCAAGGTA AATCTATGCA TATGTATTGT ATGCATCTAA CATTAATATT
 481 TTAGTATAAA TAAAAATGTG ATGTATGTAT TATTATAAAA CCTGCAATTT GAAAAAAAAG
 541 AGAGAGAGAG AGATACCCAA AACAAAAAAA GAAAACTAGT GAAAAATGCT ACAACTAAAA
 601 GAAGACTTTT CCTTAGGCCA CTATAGAAGG TTTTCTATCA CCTGTCCTCC TTGGTCCAAC
 661 TATGTCGAGA ATCGATGAAG AGCGGGCCCA GCGTCACCAA ACGGGTGTAT GGTGTGTATT
 721 CTTCTCCTCC AAAGTTGCAA TCTTGCCTCT GTGTGTGTGG TACTGTAGCG GTGCTCTTTC
 781 TTTGGTGGGT GCCACGTAGG AGAAACCCAG CAGGAGTCAT TTCCAAGTGT CACCAGTGAG
 841 AGGGCAGGAG GGCATGTGAG TTGGCGATAA CCCTATCCTC CTCCTGTCCT GGCCCTTGTC
 901 TTCTCCACGT CTCTCCCTCC CTCCCTCTCT AGAGCCTATA GCCATAGCTC ATCATCCACC
 961 CTCTCTTCCT TCCTTCCTTC CTTCCTTCCA CACAGACCAG ACCAGGCATC CCGACACTAC
1021 ACGCAGACTC TCGACTTTGT CACTAGCATC ATTGCTTGAT GATCGATGCT GAGCTGCAAC
1081 CAAGCACCAG CATATCCTTT CCTTCATTCC TTCCTGGTGC TGGTAGAAGA AGAACAAGCT
1141 AGCTAGAGTG ATAAGAGGTA ATTGTTTCCT CCAACTCATT CCAACCTTGC CTTTTGCCTG
1201 CCAAACTCCT CATCCTCTGC CACTGCATAC CATACGTGTG CGCGCCTGAA TGCCTGCCTG
1261 CCTGCCTGAT ACTGATACAT ACACACAGCA CACACGCCCC GCTATTGTAA GTGCGGCCAC
1321 CACTAGCTGT GTTTCTGCTC TCTGTGTTTA TCACCAAATT AAAGCAACGA CACTCACTTG
1381 GTCTGTTTCT TTTTAAAATA TTATTTCTCC TCCTCACCTC ACACATACCT GCATATTATT
1441 TGCAGCTAGC TACCTTGCAG ATCGATCTCC GGCCAGCGAT TGATCCCATC CAGTATAATA
```

Figure 3.6 Full length sequence of 1500bp rice *Ospsy1* promoter. The predicted TATA boxes were bolded.

(A) 1369bp *Ospsy1* promoter Fragment I

```
   1 TCTCTCAACG ATAAACTCAA CAGTATGTAA CTGAAGGGAT CAGTACTTGA GATGAAAAAG
  61 AAACAAGGAC AGAGTAGTAA AATCAGGCTA CCATCCATTT AGGGCATGTT TGGAGCAGAG
 121 TATAAAACAT GTGAATAACA CAGGAAACAA AACTTAGGAA TTTGTTTGTA TTGAAAAACA
 181 CAGCAAATTA ATGTGTATGA ACAAAAGTAA TAACAAAACA CCTGAATCAT TTTTCTCGTA
 241 TCATGTCCCT TGTGATCTTT ATTACTTCTA CTTCGATTCC ACTGGTATAA ACAGATTTTC
 301 GTTTTTGACA TTGGTATGGT ATTCAGATAG AATACAGCTT CAATAATTAA TTTATATTGT
 361 ACTAATATGT TTGTAAAGAA CTTGGTCCAA AAAAAATGTG TTTGTAAAAA CATAACGAAC
 421 ATATAATACT TTTCAAGGTA AATCTATGCA TATGTATTGT ATGCATCTAA CATTAATATT
 481 TTAGTATAAA TAAAAATGTG ATGTATGTAT TATTATAAAA CCTGCAATTT GAAAAAAAAG
 541 AGAGAGAGAG AGATACCCAA AACAAAAAAA GAAAACTAGT GAAAAATGCT ACAACTAAAA
 601 GAAGACTTTT CCTTAGGCCA CTATAGAAGG TTTTCTATCA CCTGTCCTCC TTGGTCCAAC
 661 TATGTCGAGA ATCGATGAAG AGCGGGCCCA GCGTCACCAA ACGGGTGTAT GGTGTGTATT
 721 CTTCTCCTCC AAAGTTGCAA TCTTGCCTCT GTGTGTGTGG TACTGTAGCG GTGCTCTTTC
 781 TTTGGTGGGT GCCACGTAGG AGAAACCCAG CAGGAGTCAT TTCCAAGTGT CACCAGTGAG
 841 AGGGCAGGAG GGCATGTGAG TTGGCGATAA CCCTATCCTC CTCCTGTCCT GGCCCTTGTC
 901 TTCTCCACGT CTCTCCCTCC CTCCCTCTCT AGAGCCTATA GCCATAGCTC ATCATCCACC
 961 CTCTCTTCCT TCCTTCCTTC CTTCCTTCCA CACAGACCAG ACCAGGCATC CCGACACTAC
1021 ACGCAGACTC TCGACTTTGT CACTAGCATC ATTGCTTGAT GATCGATGCT GAGCTGCAAC
1081 CAAGCACCAG CATATCCTTT CCTTCATTCC TTCCTGGTGC TGGTAGAAGA AGAACAAGCT
1141 AGCTAGAGTG ATAAGAGGTA ATTGTTTCCT CCAACTCATT CCAACCTTGC CTTTTGCCTG
1201 CCAAACTCCT CATCCTCTGC CACTGCATAC CATACGTGTG CGCGCCTGAA TGCCTGCCTG
1261 CCTGCCTGAT ACTGATACAT ACACACAGCA CACACGCCCC GCTATTGTAA GTGCGGCCAC
1321 CACTAGCTGT GTTTCTGCTC TCTGTGTTTA TCACCAAATT AAAGCAACG
```

(B) 131bp *Ospsy1* promoter Fragment II

```
  1 ACACTCACTT GGTCTGTTTC TTTTTAAAAT ATTATTTCTC CTCCTCACCT CACACATACC
 61 TGCATATTAT TTGCAGCTAG CTACCTTGCA GATCGATCTC CGGCCAGCGA TTGATCCCAT
121 CCAGTATAAT A
```

Figure 3.7 Full length sequence of 1369bp *Ospsy1* promoter Fragment I (A) and 131bp *Ospsy1* promoter Fragment II (B). The predicted TATA boxes were bolded.

3.4.2.1.1 Genomic DNA extraction

Rice *Ospsy1* promoter was cloned by PCR using genomic DNA extracted from wild type rice *Japonica* cultivar 9983 leaves using the CTAB method. Plant material and procedures employed were the same as those mentioned in Section 3.4.1.3.1.

3.4.2.1.2 Amplification of full length and fragments of Ospsy1 promoter

The full length *Ospsy1* promoter (Fragments I + II), partial *Ospsy1* promoter Fragment I and Fragment II were cloned by PCR using genomic DNA extracted (Section 3.4.1.3.1) as the DNA template.

In the cloning of the 1500bp full length *Ospsy1* promoter (Fragments I + II), a 5' EcoRI and a 3' NcoI restriction sites were introduced to flank the promoter through PCR amplification. A 25µl PCR reaction mixture containing 3µg genomic DNA, 1X High Fidelity PCR buffer, 0.2mM dNTPs, 1mM $MgSO_4$, 0.5µM forward primer B01, 0.5µM reverse primer B04 (Table 3.2) and 1 unit of Platinum® *Taq* High Fidelity polymerase (Invitrogen) was prepared. The PCR conditions were as follow: 94°C for 10 minutes, then 30 cycles at 94°C for 1 minute, 50 °C for 1 minute and 72°C for 150 seconds, followed by 1 cycle at 72°C for 7 minutes. The reaction was maintained at 15°C after cycling.

As for the cloning of partial promoter fragments, a 5' EcoRI and a 3' KpnI restriction sites were introduced to flank the 1369bp Fragment I by PCR while a 5' Hind

III and a 3' NcoI restriction sites were added to flank the 131bp Fragment II through PCR. The PCR reaction mixtures and PCR conditions for amplifying Fragment I and Fragment II were the same as that used for the full length *Ospsy1* promoter as mentioned above while the primers used in cloning Fragment I were B01 and B02, and those for cloning Fragment II were B03 and B04 (Table 3.2).

The amplified fragments were ligated into pGEM®-T vector and the sequence fidelity of the amplified fragment was confirmed by DNA sequencing (Section 3.4.3).

3.4.2.2 Cloning of *CaMV35S* minimal promoter

CaMV35S minimal promoter was cloned from pBI121 vector. Materials and methods used in plasmid preparation were the same as those mentioned in Section 3.4.1.2.1.

The *CaMV35S* minimal promoter was referred to the 100bp core promoter, as shown in Figure 3.8, containing the TATA box.

```
  1 GTGATATCTC CACTGACGTA AGGGATGACG CACAATCCCA CTATCCTTCG CAAGACCCTT
 61 CCTCTATATA AGGAAGTTCA TTTCATTTGG AGAGAACACG
```

Figure 3.8 Full length sequence of 100bp *CaMV35S* minimal promoter. The TATA box was bolded.

3.4.2.2.1 Amplification of CaMV35S minimal promoter

In the cloning of the 100bp *CaMV35S* minimal promoter, a 5' HindIII and a 3' NcoI restriction sites were introduced to flank the promoter by PCR. The PCR reaction mixtures and PCR conditions for amplifying were the same as that used for the full length *CaMV35S* promoter as mentioned in Section 3.4.1.2.2 while the primers used here were B05 and B06 (Table 3.2).

The amplified fragment was ligated into pGEM®-T vector and the sequence fidelity of the amplified fragment was confirmed by DNA sequencing (Section 3.4.3).

3.4.2.3 Cloning of rice Gt-1 promoter

The rice Gt-1 promoter used in this part was the same as that in Section 3.4.1.3, with the same restriction enzyme cutting sites 5' PstI and 3' KpnI flanking the 1801bp promoter.

3.4.2.3.1 Genomic DNA extraction

The plant material and genomic DNA preparation procedures were the same as those used in Section 3.4.1.3.1.

3.4.2.3.2 Amplification of Gt-1 promoter

For the method and procedure of PCR amplification for cloning the rice Gt-1 promoter, refer to Section 3.4.1.3.2.

3.4.2.4 Annealing of linker

A double strand linker was prepared by annealing two oligos, B11 and B12 (Table 3.2). First, 10μl of 100μM B11 was mixed with 10μl of 100μM B12 in STE buffer (10mM Tris, 50mM NaCl and 1mM EDTA, pH 8.0). The mixture was heated at 95 °C for 5 minutes and cooled down gradually at room temperature for an hour, so that the two oligos could anneal together forming the double strand linker for later use.

3.4.2.5 Making of rice GCN4 multimers

Before making the GCN4 multimers, the GCN4 monomer was prepared by annealing two oligos, B09 and B10 (Table 3.2).

Same annealing procedures mentioned in Section 3.4.2.4 were also employed in making the rice GCN4 monomer.

After annealing, phosphate groups were added to the 5' end of the monomer using T4 polynucleotide kinase (New England Biolabs, Inc.). A 25μl reaction mixture was prepared which contained 21.5μl of annealing product, 1X T4 ligase buffer and 1 unit of T4 polynucleotide kinase. The reaction mixture was incubated at 37 °C for 30 minutes.

In order to ligate the GCN4 monomers to the pGEM®-T vector, A-tailing was carried out using GoTaq® DNA polymerase (Promega). A 25μl reaction mixture containing 10μl phosphorylated GCN4 monomer, 1X GoTaq® reaction buffer, 0.2mM dATP, 2mM $MgCl_2$, 0.5μM B09, 0.5μM B10 and 1 unit of GoTaq® DNA polymerase

(Promega) was prepared. The mixture was incubated at 72°C for 30 minutes.

After A-tailing, the GCN4 monomers were ligated into pGEM®-T vector. PCR was performed to screen for vectors containing GCN4 multimers using T7 and SP6 primers, flanking the insertion site of the pGEM®-T vector.

Sequence fidelity and the copy numbers of the GCN4 monomer were confirmed by DNA sequencing (Section 3.4.3).

3.4.2.6 Construction of gene cassettes for promoter analysis

In order to study the activity of the modified promoter and transfer the gene cassettes into the rice's genome, the promoters have to be cloned into the multiple cloning site of pSB130/GUS vector to drive the expression of the GUS reporter gene.

To transfer the *CaMV35S* minimal promoter into pSB130/GUS, the 5' HindIII and 3' NcoI flanked 100bp *CaMV35S* minimal promoter, generated through PCR amplification as mentioned above, was excised by restriction digestion with HindIII and NcoI, and ligated to pSB130/GUS, which had also been cut by the same enzymes forming the pSB130/ $35S_{pro}m$/GUS

Similar procedures were carried out for putting the full length 1500bp *Ospsy1* promoter, the partial promoter fragments Fragment I and Fragment II into the pSB130/GUS vector. The full length 1500bp *Ospsy1* promoter was excised by restriction digestion with EcoRI and NcoI; Fragment I was excised by EcoRI and KpnI,

the Fragment II was excised by HindIII and NcoI; then they were ligated to pSB130/GUS vector which had been cut by the same set of enzymes respectively, giving the pSB130/*Ospsy1*$_{pro}$/GUS, pSB130/*Ospsy1(II)*$_{pro}$/GUS and pSB130/*Ospsy1(I+II)*$_{pro}$/GUS.

The pSB130/*35S*$_{pro}$*m*/GUS, pSB130/*Ospsy1(II)*$_{pro}$/GUS and pSB130/*Ospsy1(I+II)*$_{pro}$/GUS vectors were subjected to further restriction digestion to make them ready to take up the GCN4 multimers. These vectors were digested with SalI and SmaI, which were located in the multiple cloning site of the pSB130/GUS vector; thus, the opened vectors were having a sticky SalI end and a blunt SmaI end.

On the other hand, GCN4 multimers were excised using the restriction sites on the pGEM®-T vector, NcoI and SalI. First of all, the GCN4 multimers containing pGEM®-T vector was digested with NcoI. The digested product was subjected to later step in which the sticky end of NcoI digestion was removed by Klenow Fragment (3' - 5' exo) (New England Biolabs, Inc.) in 0.67mM dNTPs and 1X of working buffer. The reaction mixture was incubated at room temperature, and was halted at 75 °C for 20 minutes. The GCN4 multimer was finally excised by SalI. Thus, the GCN4 multimer was having a sticky SalI end and a blunt NcoI end; and was then ligated to the opened pSB130/*35S*$_{pro}$*m*/GUS, pSB130/*Ospsy1(II)*$_{pro}$/GUS and pSB130/*Ospsy1(I+II)*$_{pro}$/GUS vectors.

The overall cloning strategy of making the five constructs for promoter study was shown in Figure 3.9.

(A) Transferring different promoters and promoter fragments into pSB130/GUS

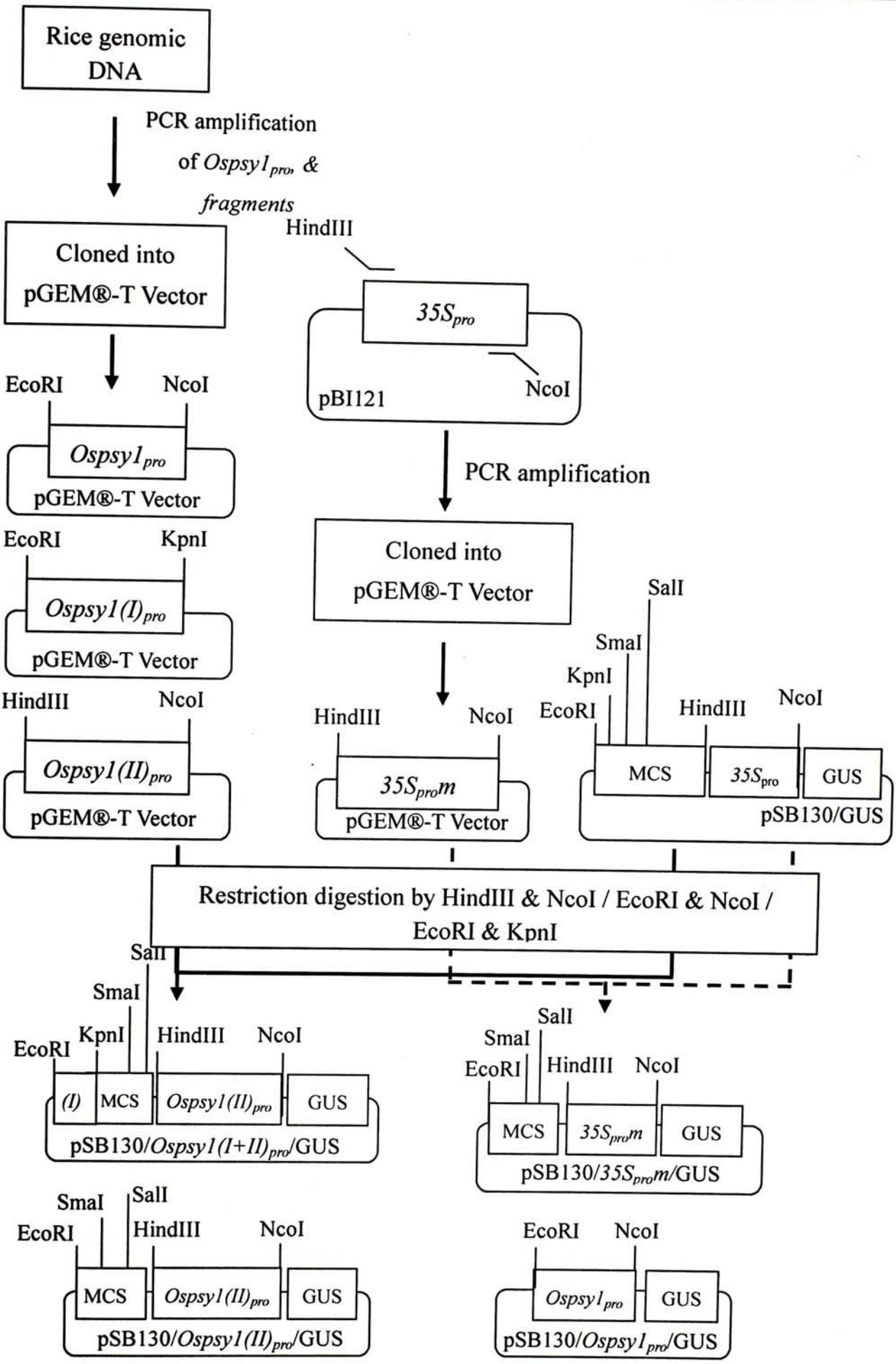


Figure 3.9 Construction of gene cassettes for promoter analysis

(A) Transferring different promoters and promoter fragments into pSB130/GUS

(B) Transferring GCN4 multimer into pSB130/GUS with different promoters

(B) Transferring GCN4 multimer into pSB130/GUS with different promoters

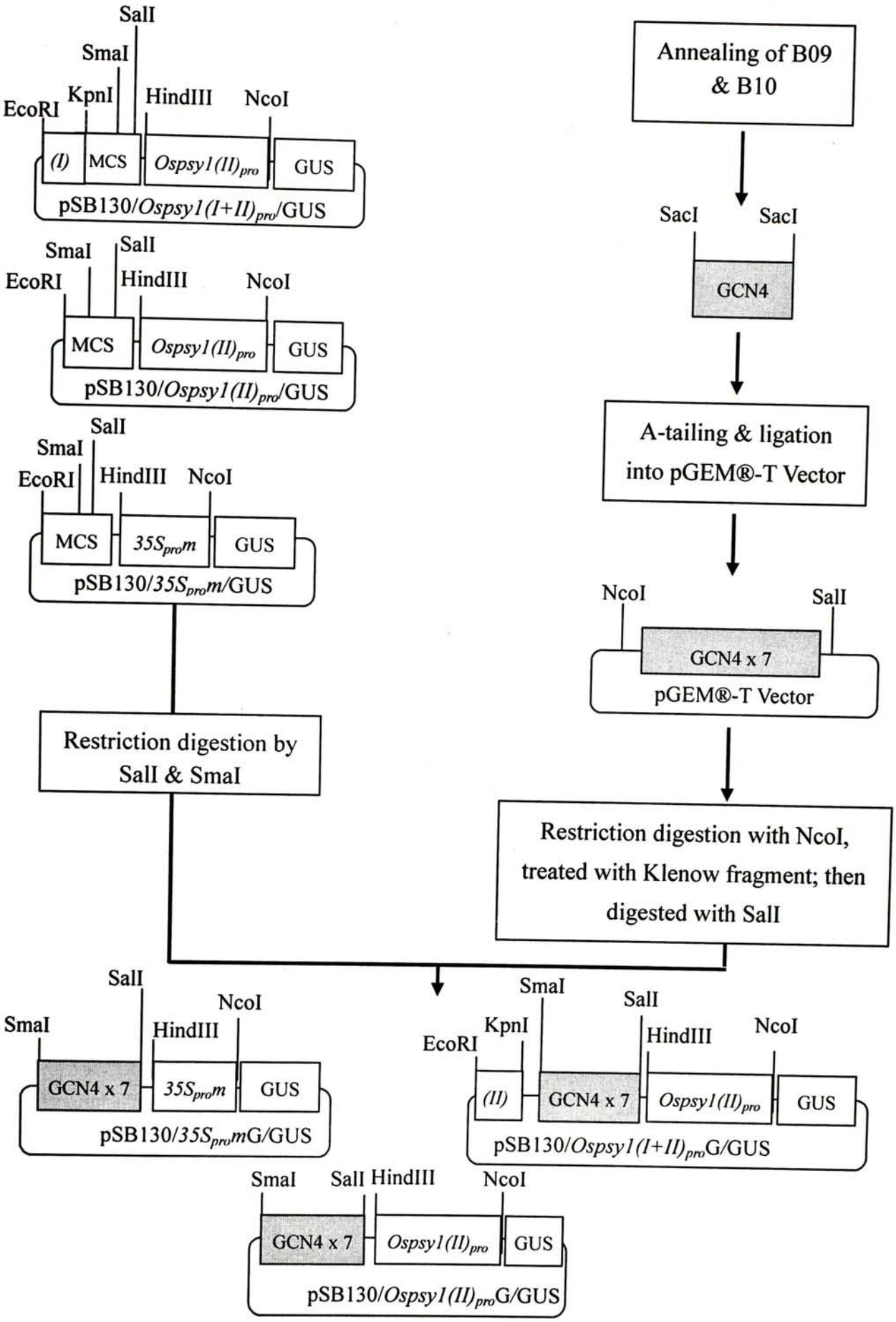


Figure 3.9 Construction of gene cassettes for promoter analysis

(A) Transferring different promoters and promoter fragments into pSB130/GUS

(B) Transferring GCN4 multimer into pSB130/GUS with different promoters

3.4.2.7 Construction of gene cassettes for Gt-1 promoter analysis

In order to study the activity of rice Gt-1 promoter in rice calli and leaves, a gene cassette was designed in which a GUS reporter gene was driven by the Gt-1 promoter.

Before ligation with the Gt-1 promoter, the pSB130/GUS vector was digested with EcoRI and NcoI and then ligated with the double strand linker prepared in Section 3.4.2.4, forming the pSB130/Linker/GUS vector. By adding the linker, a PstI and a KpnI restriction sites were introduced to the vector. Then, the pSB130/Linker/GUS was digested with PstI and KpnI, giving sticky ends to match with the Gt-1 promoter excised in the subsequent step.

On the other hand, the Gt-1 promoter was excised from the pGEM®-T vector by restriction digestion with PstI and KpnI as the two restriction sites had been introduced through PCR previously. The excised Gt-1 promoter was then ligated to pSB130/Linker/GUS forming pSB130/Gt-1_{pro}/GUS.

The overall cloning strategy for making this construct for Gt-1 promoter study was shown in Figure 3.10.

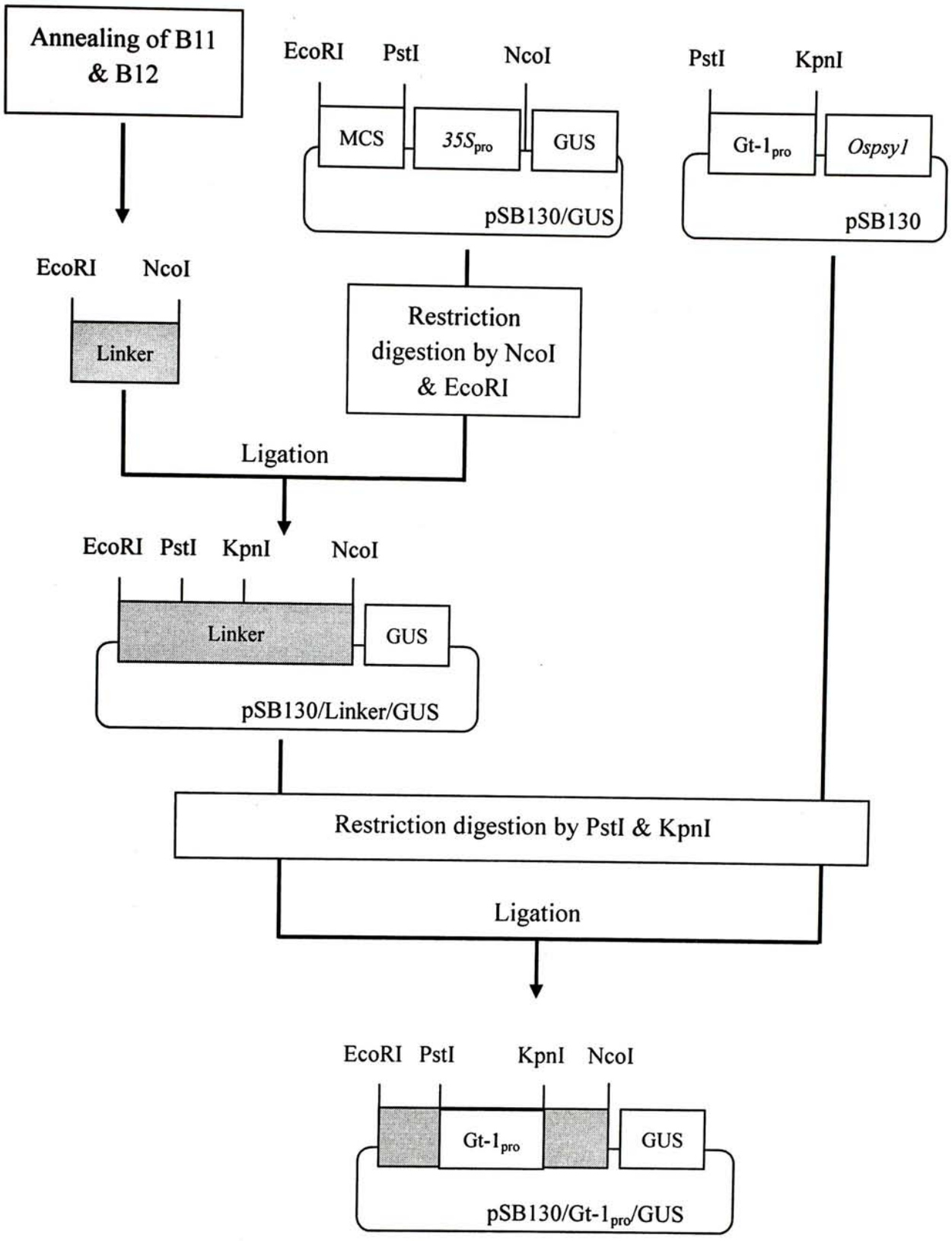


Figure 3.10 Construction of gene cassettes for Gt-1 promoter study

3.4.3 Confirmation of sequence fidelity

Sequence fidelity of all of the promoters and transgenes used in the construction of gene cassettes mentioned above were checked by cycle sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems). After purification, the amplified products were subjected to ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) for sequence analysis. All the steps carried out followed the user manual. Only constructs with correct sequences were used for *Agrobacterium* transformation.

Primers used in cloning of promoters and cDNAs were also used to check the sequence fidelity by DNA sequencing. Table 3.3 shows the additional primers used for DNA sequencing.

Table 3.3 Primer list for DNA sequencing

Name	Target	Sequence	Length
S01	***Ospsy1***	5'-AGATCTGCGAGGAGTATGCC-3'	(20mer)
S02	***Ospsy1***	5'-CGAGTCGAAGGCAACAACTG-3'	(20mer)
S03	**Gt-1$_{pro}$**	5'-CATTTGAAAAAAATATTGTA-3'	(20mer)
S04	**Gt-1$_{pro}$**	5'-TCAAGAACACAGCCTTAACC-3'	(20mer)
S05	**Gt-1$_{pro}$**	5'-GTGTATCCTCGATGAGCCTC-3'	(20mer)
S06	**35S$_{pro}$**	5'-TGTCTACTCCAAAAATATCA- 3'	(20mer)
S07	***Ospsy1$_{pro}$***	5'-TTTGTAAAAACATAACGAAC-3'	(20mer)
S08	***Ospsy1$_{pro}$***	5'-AGAAACCCAGCAGGAGTCAT-3'	(20mer)
S09	**GUS**	5'-ATGCCCACAGGCCGTCGAGTT-3'	(21mer)

3.5 Rice transformation

3.5.1 Plant materials

Mature *Oryza sativa* subsp. *Japonica* seeds, both cultivar 9983 and WYJ3, were kindly provided by Dr. Liu, Q.Q. (Agricultural College of Yangzhou University, China).

3.5.2 Preparation of *Agrobacterium*

Gene constructs (Section 3.4) were transformed into *Agrobacterium* EHA105 by freeze-thaw method. Plasmid DNA (10μg) was mixed with 250μl *Agrobacterium* competent cells. The mixture was firstly kept on ice for 5 minutes, and then dipped into liquid nitrogen for 5 minutes. After that, the mixture was incubated at 37°C for another 5 minutes. LB broth (750μl) was added into the culture for recovery, and was incubated at 28 °C with shaking at 500 rpm for 4 hours. Five hundred microlitre of the bacterial culture was spread onto a LB plate with 50μg/ml kanamycin and 25μg/ml rifampicin. The plate was incubated at 28 °C for 2 days. Single colony was picked and inoculated into 3ml of LB broth with the same antibiotic composition as the LB plate at 28 °C for 1 day. Five hundred microlitres of the transformed cells were kept as stock with 50% glycerol and stored at - 80 °C, the rest of them were used for minipreparation. PCR was performed for plasmid verification to ensure successful transformation.

Agrobacterium culture harbouring desired construct was freshly prepared by streaking the stock on LB plate with antibiotics mentioned above. Single colony was picked and inoculated into 3ml LB with appropriate antibiotics at 28 °C for 1 day. One hundred microlitre of the bacterial culture was inoculated into 10ml LB in the day after and was kept at 28 °C for 16 hours. Just before co-cultivation, the bacterial culture was pelleted by centrifugation at 3,341 rcf for 5 minutes and resuspended in 10ml 10mM $MgSO_4$. The optical density (OD_{600}) of the culture was taken. After that, the culture was centrifuged again and resuspended in 10-15 ml AA medium (Table 3.4) so that the *Agrobacterium* suspension was about 0.2 in OD_{600}, and was ready for rice transformation.

3.5.3 *Agrobacterium* mediated transformation

The media (Table 3.4) and methods used for rice transformation followed the one described by Liu (2002), and modified by J.J. Lee (The Chinese University of Hong Kong, Hong Kong).

3.5.4 Callus induction from mature rice seeds

The husks of mature rice seeds were first removed. Seeds were transferred into 250ml conical flask and washed with 100ml 70% ethanol for 1 minute with continuous shaking, and then sterilized in 100ml 50% Clorox for 90 minutes at room temperature with continuous shaking at 250 rpm. Sterilized seeds were rinsed with sterilized water for 5 to 10 times and blotted dry. Sterilized seeds were placed on primiary calli induction medium (Table 3.4) and cultivated in dark at 28 °C for 3 weeks.

3.5.6 Co-cultivation and selection

Calli induced were separated from the seeds and cultivated on the same induction medium overnight. On the next day, induced calli were cleaned and dried with sterilized filter paper; and were then immersed in the *Agrobacterium* culture prepared (Section 3.5.2) for 15 minutes with occasional shaking. After blotting dry, calli were transferred onto co-cultivation medium (Table 3.4) and kept at 28 °C for 3 days in dark.

After co-cultivation, calli were blotted dried and transferred onto selection medium (Table 3.4) supplemented with 500μg/ml cefotaxime and 50μg/ml hygromycin. The calli were cultivated at 28 °C in darkness for 2 to 3 months, and subcultured every 2 weeks.

3.5.7 Pre-regeneration and regeneration of transgenic rice

Yellowish resistant calli were transferred onto pre-regeneration medium (Table 3.4) supplemented with 50μg/ml hygromycin. The calli were placed in dark at 28 °C for a week and then were transferred into growth chamber with a 16/8 hour day/night cycle at 25 °C in the subsequent week. After pre-regeneration, the calli were transferred onto regeneration medium (Table 3.4) and placed in growth chamber at 25°C with a 16/8 hour day/night photoperiod until rice plantlets were formed.

3.5.8 Plantation of transgenic rice

Rice plantlets were separated from the regeneration medium. After washing, the plantlets were placed in tap water for 1 to 3 weeks before plantation. All the transgenic rice in this project was grown in Gene Garden (The Chinese University of Hong Kong, Hong Kong).

Table 3.4 Media used in rice transformation

Name of media	Composition
AA medium	AA (Toriyama and Hinata, 1985) nutrients, MS medium, casein hydrolase 0.5g/L, glucose 10g/L, sucrose 30g/L, AS (acetosyrigone) 100 mol/L, 2,4-D 2mg/L, pH6.0
Primiary calli induction medium	Higrow® Rice medium (GIBCO-BRL), gelrite 3.3g/L
N_6D_2	N_6 (Chu,1978) nutrients and vitamins, casein hydrolase 0.5g/L, sucrose 30g/L, 2,4-D 2mg/L, pH 6.0
Co-cultivation medium	N_6D_2, glucose 10g/L, AS 100 mol/L, gelrite 3.0g/L, pH 6.0
Selection medium	N_6D_2, cefotaxime 500μg/ml, hygromycin B 50μg/ml, gelrite 3.5g/L, pH 6.0
Pre-regeneration medium	Higrow® Rice medium (GIBCO-BRL), hygromycin B 50μg/ml, gelrite 3.3g/L
Regeneration medium	N_6 (Chu,1978) nutrients and vitamins, hygromycin B 50μg/ml , casein hydrolase 1g/L, maltose 30g/L, kinetin 2mg/L, NAA (1-naphthylacetic acid) 1mg/L , gelrite 6g/L, pH 5.9

3.6 Detection of transgene expression

3.6.1 Detection at DNA level

3.6.1.1 Genomic DNA extraction

In order to confirm the insertion and the copy number of transgene in the genome, genomic DNA was extracted from the resistant calli and leaves of the transgenic rice. Resistant calli used in this part was freeze-dried beforehand, and 0.5 gram of the dried calli was used for extraction while one gram of fresh transgenic rice leaves was used in extraction. The genomic DNA preparation procedures were same as those used in Section 3.4.1.3.1. The final genomic DNA obtained was treated with 1ng RNaseA and incubated at 37 °C for an hour to prevent RNA contamination. Genomic DNA concentration was determined by OD_{260} measurement with a spectrophotometer while the quality of DNA was checked by gel electrophoresis in a 0.8% agarose/TAE (0.04M Tris-acetate, 1μM EDTA and 0.5mg/L ehtidium bromide) gel.

3.6.1.2 PCR screening

To screen for transgenic rice having the target gene cassettes, PCR screening was performed using genomic DNA extracted from resistant rice calli and T_0 rice leaves. A 25μl PCR reaction mixture was prepared containing 2 - 5μg genomic DNA, 1X GoTaq® reaction buffer, 0.2mM dNTPs, 1 - 4mM $MgCl_2$, 0.5μM forward primer,

0.5μM reverse primer and 1 unit of GoTaq® DNA polymerase (Promega). The $MgCl_2$ concentration used in PCR reaction varied with different amplified targets. The PCR conditions were as follow: 94°C for 10 minutes, then 30 cycles at 94°C for 1 minute, 55 °C for 1 minute and 72°C for extension, followed by 1 cycle at 72°C for 7 minutes. The reaction was maintained at 15°C after cycling. The extension time was adjusted based on the length of different amplified targets.

Primers used for PCR screening of different gene cassettes were listed in the following Table 3.5.

Table 3.5 Primer list for Genomic DNA PCR screening

Name	Target	Sequence	Length
P01	**pSB130/*35S*$_{pro}$/*Ospsy1***	5' -TGTGGTGTAGGAGGACAGATGAGCTTG- 3'	(27mer)
P02	**pSB130/*35S*$_{pro}$/*Ospsy1***	5' -TCCTCTCCAACGTCACGGAGTATATTT- 3'	(27mer)
P01	**pSB130/Gt-1$_{pro}$ /*Ospsy1***	5' -TGTGGTGTAGGAGGACAGATGAGCTTG- 3'	(27mer)
P02	**pSB130/Gt-1$_{pro}$ /*Ospsy1***	5' -TCCTCTCCAACGTCACGGAGTATATTT- 3'	(27mer)
P03	**pSB130/*35S*$_{pro}$/*Ospsy2***	5'-GCGCGCGGTACCATGGCGTCCTCCTCGTCG GCGGCG-3'	(36mer)
P04	**pSB130/*35S*$_{pro}$/*Ospsy2***	5'-CATAGATTGCCCAGACAGCTTTGCGCC- 3'	(27mer)
P03	**pSB130/Gt-1$_{pro}$ /*Ospsy2***	5'-GCGCGCGGTACCATGGCGTCCTCCTCGTCG GCGGCG-3'	(36mer)
P04	**pSB130/Gt-1$_{pro}$ /*Ospsy2***	5'-CATAGATTGCCCAGACAGCTTTGCGCC- 3'	(27mer)
B13	**pSB130/*35S*$_{pro}$/GUS**	5'-ATGGTCCGTCCTGTAGAAACCCCAACC-3'	(27mer)
B14	**pSB130/*35S*$_{pro}$/GUS**	5' -TAGTGCCTTGTCCAGTTGCAACCACCT-3'	(27mer)
B15	**pSB130/ *35S*$_{pro}$*m*/GUS**	5' -ATGGTCCGTCCTGTAGAAACCCCAACC-3'	(27mer)
B16	**pSB130/ *35S*$_{pro}$*m*/GUS**	5' -CGCAGGTGATCGGACGCGTCGGGTCGA-3'	(27mer)

Table 3.5 Primer list for Genomic DNA PCR screening (Continued)

Name	Target	Sequence	Length
B15	**pSB130/*35S*$_{pro}$ *mG*/GUS**	5' -ATGGTCCGTCCTGTAGAAACCCCAACC-3'	(27mer)
B16	**pSB130/*35S*$_{pro}$ *mG* /GUS**	5' -CGCAGGTGATCGGACGCGTCGGGTCGA-3'	(27mer)
B13	**pSB130/*Ospsy1* $_{pro}$/ GUS**	5'-ATGGTCCGTCCTGTAGAAACCCCAACC-3'	(27mer)
B14	**pSB130/*Ospsy1* $_{pro}$/ GUS**	5' -TAGTGCCTTGTCCAGTTGCAACCACCT-3'	(27mer)
B13	**pSB130/*Ospsy1* (I+II)$_{pro}$G/ GUS**	5'-ATGGTCCGTCCTGTAGAAACCCCAACC-3'	(27mer)
B14	**pSB130/*Ospsy1* (I+II)$_{pro}$G/ GUS**	5' -TAGTGCCTTGTCCAGTTGCAACCACCT-3'	(27mer)
B13	**pSB130/GCN4/ *Ospsy1(II)*$_{pro}$G/ GUS**	5'-ATGGTCCGTCCTGTAGAAACCCCAACC-3'	(27mer)
B14	**pSB130/GCN4/ *Ospsy1(II)*$_{pro}$G/ GUS**	5' -TAGTGCCTTGTCCAGTTGCAACCACCT-3'	(27mer)
B13	**pSB130/Gt-1$_{pro}$ / GUS**	5'-ATGGTCCGTCCTGTAGAAACCCCAACC-3'	(27mer)
B14	**pSB130/Gt-1$_{pro}$ / GUS**	5' -TAGTGCCTTGTCCAGTTGCAACCACCT-3'	(27mer)

3.6.1.3 Synthesis of DIG-labeled DNA probes

Double-stranded DIG-labeled DNA probes were synthesised by using DIG DNA Labelling Kit (Roche Applied Science) from two rounds of PCR. In the first round PCR, the 400 - 700bp templates for making the probes were prepared by a 25μl PCR reaction mixture containing 1 - 2μg of pGEM®-T vector with the *Ospsy1* and *Ospsy2* cDNA, and the pSB130/GUS vector respectively, 1X GoTaq® reaction buffer, 0.2mM dNTPs, 1 - 4mM $MgCl_2$, 0.5μM forward primer, 0.5μM reverse primer and 1 unit of GoTaq® DNA polymerase (Promega). The $MgCl_2$ concentration used in PCR reaction varied with different amplified targets. The PCR conditions were as follow: 94°C for 10 minutes, then 30 cycles at 94°C for 1 minute, 58 °C for 1 minute and 72°C for 2 minutes, followed by 1 cycle at 72°C for 7 minutes. The reaction was maintained at 15°C after cycling. After that, the PCR amplified products were recovered for the second round PCR.

In the second round PCR, a 100μl PCR reaction mixture containing 50ng first round PCR products as DNA template, 1X GoTaq® reaction buffer, 0.02mM DIG-labeled dNTPs, 2mM $MgCl_2$, 0.5μM forward primer, 0.5μM reverse primer and 5 units of GoTaq® DNA polymerase was prepared. The PCR conditions were as follow: 94°C for 5 minutes, then 55 cycles at 94°C for 45 seconds, 58 °C for 1 minute and 72°C for 2 minutes, followed by 1 cycle at 72°C for 7 minutes. The reaction was maintained

at 15°C after cycling. The primers used for making different probes were shown in Table 3.6.

The concentrations of the probes were determined by dot blot comparing to a known amount DIG-labeled DNA control supplied in the kit (Roche Applied Science).

Table 3.6 Primer list for making DIG-labeled probes

Name	Target	Sequence	Length
P01	***Ospsy1***	5' -TGTGGTGTAGGAGGACAGATGAGCTTG- 3'	(27mer)
P02	***Ospsy1***	5' -TCCTCTCCAACGTCACGGAGTATATTT- 3'	(27mer)
P03	***Ospsy2***	5'-GCGCGCGGTACCATGGCGTCCTCCTCGTCGGC GGCG-3'	(36mer)
P04	***Ospsy2***	5'-CATAGATTGCCCAGACAGCTTTGCGCC- 3'	(27mer)
P05	**GUS**	5'-ATGGTCCGTCCTGTAGAAACCCCAACC-3'	(27mer)
P06	**GUS**	5' –TAGTGCCTTGTCCAGTTGCAACCACCT-3'	(27mer)

3.6.1.4 Southern blot analysis

Genomic DNA (15μg) was digested with suitable restriction enzyme overnight, which only has single cutting site between left and right border of the gene cassette at 37°C. Genomic DNA from the transgenic plant transformed with different gene cassettes were digested with different restriction enzymes. Those extracted from pSB130/*35S*$_{pro}$/*Ospsy1*, pSB130/Gt-1$_{pro}$/*Ospsy1*, pSB130/*35S*$_{pro}$/*Ospsy2* and pSB130/Gt-1$_{pro}$/*Ospsy2* transformed leaves were digested with BamHI.

For the transgenic rice calli, those transformed with pSB130/*35S*$_{pro}$/*Ospsy1*and pSB130/Gt-1$_{pro}$/*Ospsy1* were digested with BamHI while those transformed with pSB130/*35S*$_{pro}$/*Ospsy2* and pSB130/Gt-1$_{pro}$/*Ospsy2* were cut by PstI.

As for the promoter study, genomic DNA extracted from pSB130/*35S*$_{pro}$/ GUS, pSB130/*35S*$_{pro}$m/GUS, pSB130/*35S*$_{pro}$mG/GUS, pSB130/*Ospsy1*$_{pro}$/GUS, pSB130/ *Ospsy1(II)*$_{pro}$G/GUS and pSB130/*Ospsy1(I+II)*$_{pro}$G/ GUS were digested with NcoI.

The digested products were separated by gel electrophoresis in a 0.8% agarose/TAE gel and transferred to a positively-charged nylon membrane (Roche Applied Science) by VacuGeneXL Vacuum Blotting System (Pharmacia Biotech). All the steps carried out followed those described in the user manual. Double-stranded probe was first denatured at 99°C for 5 minutes before hybridization. Hybridization and detection were performed following the DIG Nucleic Acid Detection Kit User Manual (Roche Applied Science).

3.6.2 Detection at RNA level

In order to confirm the expression of transgene in the transgenic plants, total RNA was extracted from the 15-20 DAF immature seeds of the transgenic rice.

3.6.2.1 Total RNA extraction

The total RNA preparation procedures were the same as those mentioned in Section 3.4.1.1.1.

3.6.2.2 Northern blot analysis

Total RNA (5μg) was separated by gel electrophoresis in a 1% agarose/formaldehyde gel and transferred to a positively-charged nylon membrane (Roche Applied Science) by capillary transfer method. Double-stranded DIG-labeled DNA probe was prepared as described in Section 3.6.1.3. Hybridization and detection were performed following the procedures described in the DIG Nucleic Acid Detection Kit User Manual (Roche Applied Science).

3.6.3 Detection at protein level

3.6.3.1 Antibody production

Polyclonal *Ospsy1* and *Ospsy2* antibodies were made by immunizing rabbit with the two *Ospsy* proteins induced in *E.coli* using the pET system.

3.6.3.1.1 Ospsy1 and Ospsy2 induction in pET system

pET *E. coli* expression system was adopted to make *Ospsy* proteins. The two *Ospsy* cDNA were PCR amplified using specific primers listed in Table 3.7 with the introduced 5' NcoI and 3'EcoRI restriction sites and cloned into pGEM®-T vector. PCR procedure and conditions were the same as those mentioned in Section 3.4.1.1.2 and Section 3.4.1.1.3. After sequence fidelity confirmation, the two cDNA were excised with NcoI and EcoRI endonuclease and ligated to pET-30a vector (Novagen) (Figure 3.11).

Table 3.7 Primer list for cloning *Ospsy1* and *Ospsy2* into pET-30a

Name	Target	Sequence	Length
A09	***Ospsy1***	NcoI 5' -TACCATGGCAATGGCGCCCATCACGC- 3'	(26mer)
A10	***Ospsy1***	EcoRI 5' -AGAATTCCTACTTCTGGCTATTTCTC- 3'	(26mer)
A11	***Ospsy2***	NcoI 5' -TACCATGGCAATGGCGTCCTCCTCGTCGGCGGCG- 3'	(34mer)
A12	***Ospsy2***	EcoRI 5' -AGAATTCTCATGATGCAACTGCCGC- 3'	(25mer)

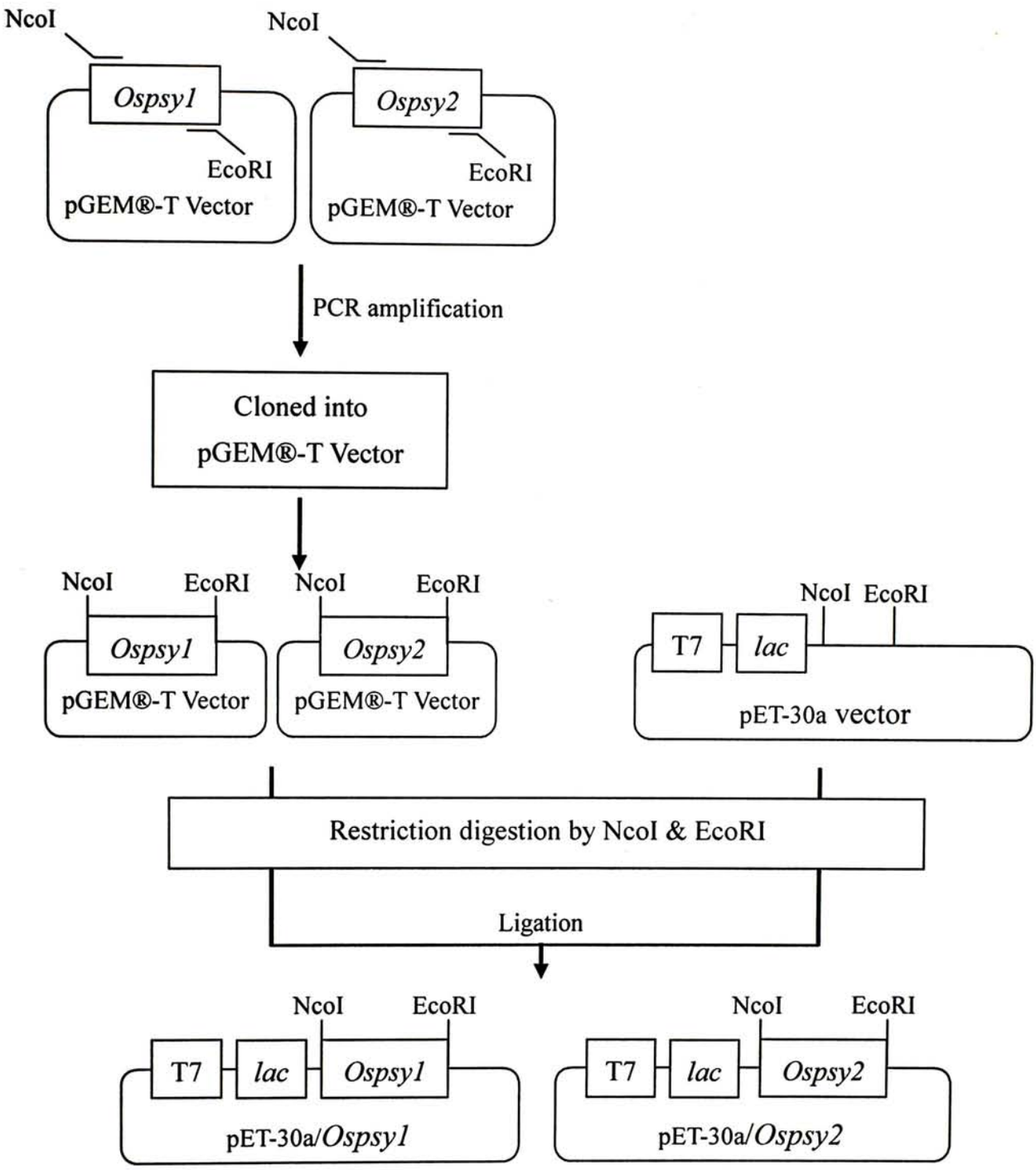


Figure 3.11 Cloning of *Ospsy1* and *Ospsy2* into pET system

E. coli strain Rosetta (2) was transformed with the pET-30a/*Ospsy1* and pET-30a/*Ospsy2* constructs. Single colony of the transformant was picked respectively and inoculated with 3ml LB with 50μg/ml kanamycin. The culture was incubated at 37°C with continuous shaking at 250 rpm for 16 hours. After that, 200μl of the cell culture was kept as stock with 50% glycerol and stored at - 80 °C, the rest of them were used for minipreparation. PCR was performed for plasmid verification to ensure successful transformation.

In the course of induced protein expression, 20μl of the glycerol stock was used to inoculate 5ml LB broth with same amount of antibiotic mentioned above. The culture was incubated at 37°C with continuous shaking at 250 rpm until the optical density (OD_{600}) of the culture reached 0.7. Then 1ml of the bacterial culture was collected as the negative control and IPTG was added to the culture to make the final concentration up to 1mM for protein induction. After 5 hours of induction at 27 °C, *E. coli* cell pellet was obtained by centrifugation at 1,485 rcf for 10 minutes. Bacterial protein was extracted with bacterial protein extraction buffer (0.1M Tris-HCl pH7.5, 0.03M NaCl, 7.75% SDS and 10% 2-mercaptoethanol) at 99 °C for 10 minutes. Bacterial protein was obtained by collecting the supernatant after centrifugation at 1,485 rcf. Tricine-SDS PAGE (Section 3.6.3.3) was carried out to separate the bacterial protein from the OsPSY1 and OsPSY2 protein respectively. The induced OsPSY proteins were directly

cut out from the gel and eluted with phosphate buffer. After two rounds of purifications, the OsPSY proteins were dissolved into 10ml of sterilized water. The amount of OsPSY proteins recovered were quantitated by comparing the intensity of know amount of BSA in tricine-SDS PAGE.

3.6.3.1.2 Immunization of rabbit and serum collection

Each antigen, OsPSY1 and OsPSY2, was used to immunize two rabbits. A total of 4 injections were performed for each rabbit. Injection was done every two weeks. In every single injection, 150μg of antigen mixed with adjuvant in a 50:50 emulsion was used to immunize the rabbits. Serum (1ml) of the immunized rabbit was collected before the first injection as a negative control, before the final injection for titer checking and, finally, two weeks after the last injection. All the immunization and serum collection procedures were carried out in the help of the technicians in Shanghai Fraternity Association Research Services Centre at the Chinese University of Hong Kong following animal ethics guideline. After collecting the serum, dot blot was performed to check the titer of each antibody.

3.6.3.2 Total protein extraction from plant materials

3.6.3.2.1 Protein extraction from rice calli and leaves

In the extraction of total protein for western blot analysis, about 0.5 gram freeze-dried rice calli and 0.5 gram of fresh rice leaves were used. The plant materials were ground into powder in liquid nitrogen. Leaf protein extraction buffer (700μl) (100mM Tris-HCL, 300mM NaCl and 1mM EDTA, pH 7.4) was added to the powder. The homogenate was kept in ice for 30 minutes. After that, the homogenate was subjected to centrifugation at 11,485 rcf at 4 °C for 15 minutes. The supernatant was collected and mixed with equal amount of protein loading buffer (100mM Tris, 24% glycerol, 8% SDS, 4% 2-mercaptoethanol and trace amount of bromophenol blue). The mixture was heated at 99 °C for 10 minutes before used in tricine SDS-PAGE.

3.6.3.2.2 Protein extraction from immature and mature rice seeds

In the extraction of total seed protein for western blot analysis, the husks of both the mature and immature rice seeds were removed, and about 0.05 gram of grain was used for extraction. The grain was ground into fine powder and 700μl seed protein extraction buffer (125mM Tris-HCl, 4M urea, 4% SDS and 5% 2-mercaptoethanol, pH 6.8) was added to the powder. The homogenate was incubated at 37°C with continuous shaking at 1400 rpm for 2 hours. After that, the homogenate was subjected to centrifugation at 11,485 rcf at room condition for 15 minutes. The supernatant was

collected and mixed with equal amount of protein loading buffer (100mM Tris, 24% glycerol, 8% SDS, 4% 2-mercaptoethanol and trace amount of bromophenol blue). The mixture was heated at 99 °C for 10 minutes before used in tricine SDS-PAGE.

3.6.3.3 Tricine SDS-PAGE

Protein samples were loaded into 16.5% tricine gel with anode buffer (0.2M Tris-base, pH 8.9) and cathode buffer (0.1M Tris-base, 0.1M Tricine and 0.1% SDS, pH 8.25) for electrophoresis. After that, tricine gel was stained with Coomassie blue solution (1 g/L Coomassie brilliant blue G-250 in methanol:100% glacial acetic acid:water, 20:6:55) and destained with destaining solution (methanol:100% glacial acetic acid:water, 20:6:55) so that the proteins could be visualized.

3.6.3.4 Western blot analysis

After tricine SDS-PAGE, protein samples were transferred onto PVDF membrane (Roche Applied Science) using the mini-Trans-blot electrophoretic transfer cell (Bio-Rad) following the procedure described in the user manual. Electroblotting was carried out at constant voltage (100V) for 90 minutes in Towbin buffer (25mM Tris, 192mM glycine, 20% methanol, 70% sterilized water, pH 8.3).

After electroblotting, the membrane was used in immunodetection using Aurora™ Western blotting kit (MP Biomedicals). All the procedures followed as described in user

manual. Both the polyclonal rabbit anti-OsPSY1 and anti-OsPSY2 antibody were used at a dilution of 1:3000. (HRP)-conjugated goat anti-rabbit immuno-globulin G (Sigma-Aldrich Corp.) was used at a dilution of 1:8000 as the secondary antibody.

3.6.4 Detection at metabolite level

3.6.4.1 Isoprenoids extraction from plant materials

All the extraction and manipulation steps were carried out on ice and shielded from light to minimize carotenoids degradation.

The extraction method was modified from that of Fraser *et al.* (Fraser *et al.*, 2000). Plant material was freeze-dried and ground into powder with mortar and pestle. To every 2mg of tissue, 100µl methanol was added and mixed by inversion for 5 minutes at 4°C. As for the leave sample, KOH was added, to make up the final concentration of 6%, to the mixture and incubated at 60 °C for 30 minutes in dark to remove chlorophyll. After that, equal volume of Tris-HCl (50mM, pH7.5 with 1M NaCl) was added and the homogenate was kept on ice for 10 minutes. Four hundreds microlitres of chloroform was added to the mixture and incubated on ice for 10 minutes after mixing by inversion for 5 minutes. Centrifugation at 3,000 rcf at 4°C at 5 minutes was performed to separate the aqueous and organic phase. The organic phase was collected and the aqueous phase was re-extracted with 400µl chloroform. The pooled chloroform extracts were dried by

centrifugal evaporation and dissolved in 150 - 200μl ethyl acetate for UPLC analysis. The extracts were filtered with LC 13 mm syringe filter with 0.2μm PVDF membrane (PALL Acrodisc ®) before analysis. UPLC identification was carried out immediately after extraction. Astaxanthin, canthaxanthin and vitamin A alcohol were used as internal standards for different plant tissues.

3.6.4.2 UPLC analysis for isoprenoid identification

ACQUITY UPLC® System (Waters) equipped with photodiode array detector was used in this study. UPLC was performed using a BEH C18 column (1.7μm, Waters) and the method was modified from Fraser *et al.* (Fraser *et al.*, 2000). Throughout the process of chromatography, the eluate was monitored continuously from 200 to 500nm and the column temperature was maintained at 15°C. Mobile phases consisting of methanol (A), water/methanol (20/80 by volume) (B) and tert-methyl butyl ether (C) were used and the flow rate of mobile phases was maintained as 0.3ml per minute. The gradient elution used was 95%A, 5% B isocratically for 2 minutes, a step to 80% A, 5% B , 15% C at 2 minute, followed by a linear gradient to 30% A, 5% B 65% C by 5 minutes. A period of 30 seconds was used to return the column to the initial concentration of A and B. Elution of carotenoids was followed at 260nm and 477nm. The identities of the carotenoids were identified by comparing their UPLC profile with the standards' profile.

3.6.5 Detection of promoter activity

3.6.5.1 Histochemical staining

3.6.5.1.1 Histochemical staining of GUS activity

Fresh rice calli, leaves and 15 – 20 DAF immature seeds were used in GUS histochemical staining. All immature seeds were dissected transversally by hand before staining. All the solution and procedure employed follow those described by Jefferson *et al.* (Jefferson *et al.*, 1987). Five milliliter GUS staining solution (0.1M sodium phosphate buffer, 0.1% triton X-100, 1mM EDTA, 0.5mM $K_3Fe(CN)_6$, 0.05mM $K_4Fe(CN)_6$, 0.05% X-glu, pH 7.0) was added to the plant tissue and was incubated at 37 ^{o}C in darkness for 24 hours for blue foci to developed which indicating GUS activity. After staining, the sections were rinsed in 70% ethanol for 5 minutes.

3.6.5.1.2 Plant tissue fixation for microscopic observation

Transverse sections of stained immature rice seeds were first fixed with FAA solution (50% ethanol, 10% formaldehyde, 5% acetic acid and 35% sterilized water) for 16 hours in room condition. After rinsing with 50% ethanol for 5 times with 15 minutes each, the stained seeds were subjected to subsequent dehydration, clearing and infiltration of paraffin wax (McCormic Scientific) using the Enclosed Tissue Processor (Leica TP-1050). Finally, sections were embedded into paraffin wax and the blocks

were allowed to solidify in room condition for 2 hours. After microtome sectioning of the paraffin block with the seeds sample to about 5 um think, the sample was mounted on glass slide for observation under fluorescent microscope (Nikon Microscope Eclipse 80*i*).

3.6.5.2 GUS activity assay

3.6.5.2.1 Protein extraction and quantitation with Bio-Rad protein assay

Fresh rice leaves and 15 – 20 DAF immature seeds were used in GUS activity assay. All the solution and procedure employed follow those described by Jefferson *et al.* (Jefferson *et al.*, 1987).

Total protein of the plant materials was ground in 500µl GUS extraction buffer (50mM sodium phosphate buffer, 10mM EDTA, 0.1% triton X-100 and 0.1% 2-mercaptoethanol, pH 7.0). The homogenate was kept on ice for 20 minutes. Supernatant was collected after centrifugation at 11,485 rcf for 10 minutes. Protein concentration of the extract was determined based on the Bradford method using BioRad Protein Assay following the procedures described in the user manual. The Bio-Rad Protein Assay Dye reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts sterilized water. Ten microlitres of plant extract was mixed with 180µl diluted dye reagent. The mixture was incubated at room temperature for 15

minutes. Absorbance of the mixture at 595nm was measured with Tecan Safire spectrofluorometer. The protein concentration of the plant extract was determined by comparing its absorbance with that of the BSA standard.

3.6.5.2.2 GUS activity assay

Fluorometric assay was performed as described by Jefferson *et al.* (Jefferson *et al.*, 1987). Forty microlitres assay buffer (1mM MUG in GUS extraction buffer) was added to 10µl plant extract. The mixture was incubated at 37 °C in darkness for 30 minutes. 25 microlitres of stop buffer (0.2M Na_2CO_3) was added to the mixture to halt the reaction. Fluorescence signal was measured with excitation at 365nm, emission at 455nm with Tecan Safire spectrofluorometer. GUS activity of the plant extract was determined by comparing its fluorescence signal with those emitted by the 4-MUG standards.

Chapter 4: Results

4.1 Tissue-specificity and endosperm specific expression of rice *psy1* and *psy2*

In order to determine the tissue-specific activities of rice phytoene synthase enzymes, OsPSY1 and OsPSY2 and their potential for making Golden Rice, the following four gene cassettes were constructed, as depicted in Figure 4.1.

In $35S_{pro}$/*Ospsy1* and $35S_{pro}$/*Ospsy2*, the expression of rice phytoene synthases was controlled under the constitutive promoter. If OsPSY1 and OsPSY2 activities are tissue-specific, the overexpression of the OsPSYs would only affect the carotenoids level in specific tissues. Therefore, by monitoring the changes in carotenoids levels in leaf and seed of the transgenic rice, the tissue-specific activity of OsPSY1 and OsPSY2 can be revealed. On the other hand, in Gt-1_{pro}/ *Ospsy1* and Gt-1_{pro}/ *Ospsy2*, the two rice phytoene synthases were expressed in the rice endosperm under the control of the seed-specific Gt-1_{pro}. A change of the phytoene level in transgenic rice seed was expected. By determining the change of phytoene in transgenic seeds, we can find out a suitable candidate for making Golden Rice.

The four cassettes were transformed into rice calli of *Japonica* WYJ3, and regenerated transgenic plants were subjected to the following analysis. First of all, genomic DNA PCR and Southern blot analysis were employed to screen for positive

transformants and to check the copy number of transgene in genome of the transgenic rice. Then, northern blot and western blot were carried out to determine the expression of transgene in different rice tissues. The OsPSY-specific antibodies were prepared by immunizing rabbits with OsPSY1 and OsPSY2 proteins produced in bacterial system. The activities of the two OsPSY enzymes were determined by monitoring the changes in carotenoids levels in different rice tissues by ultra-performance liquid chromatography (UPLC) analysis. And the amount of different carotenoids species in rice tissues were calculated with the help of internal and external carotenoids standards.

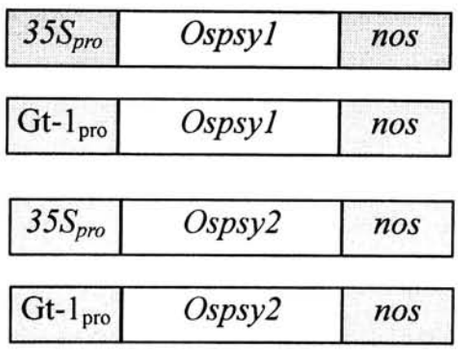


Figure 4.1 Schematic diagram of gene cassettes constructed for *Ospsy* genes

Abbreviations:

35S$_{pro}$	:	CaMV35S promoter	*Ospsy1*	:	Rice phytoene synthase 1
Gt-1$_{pro}$	:	Glutelin-1 promoter	*Ospsy2*	:	Rice phytoene synthase 2
nos	:	nos			

4.1.1 Construction of gene cassettes for study on *Ospsy1* and *Ospsy2*

All of the two promoters and two cDNAs involved were cloned successfully, as shown in Figure 4.2(A). *CaMV35S*$_{pro}$ was cloned from pBI221 using primer sets A05 and A06, while *Ospsy2* was amplified using primer sets A03 and A04 from J033025L21. The *Ospsy1* cDNA was obtained by RT-PCR using primer sets A01 and A02, and the Gt-1$_{pro}$ was cloned using primer sets A07 and A08 by PCR with total RNA and genomic DNA from wild type *Japonica* 9983 leaves respectively. The expected sizes of the four fragments were 801bp for *CaMV35S*$_{pro}$, 1861bp for Gt-1$_{pro}$, 1263bp for *Ospsy1* and 1197bp for *Ospsy2*. Then, the fragments were cloned into pSB130 vector to give the four constructs. After transformation into *Agrobacterium,* the four constructs were isolated by mini-preparation and PCR amplification was performed to screen for positive transformant. The full length of *35S*$_{pro}$*/Ospsy1*, Gt-1$_{pro}$*/Ospsy1* and *35S*$_{pro}$*/Ospsy2* were amplified successfully, using primer sets A05 and A02, A07 and A02, A05 and A04, with the expected sizes of 2064bp, 3124bp and 1998bp respectively. However, full length amplification of Gt-1$_{pro}$*/Ospsy2* was not successful; instead, the promoter and cDNA fragments with the expected sizes of 1861bp and 1197bp were amplified separately, as depicted in Figure 4.2(B).

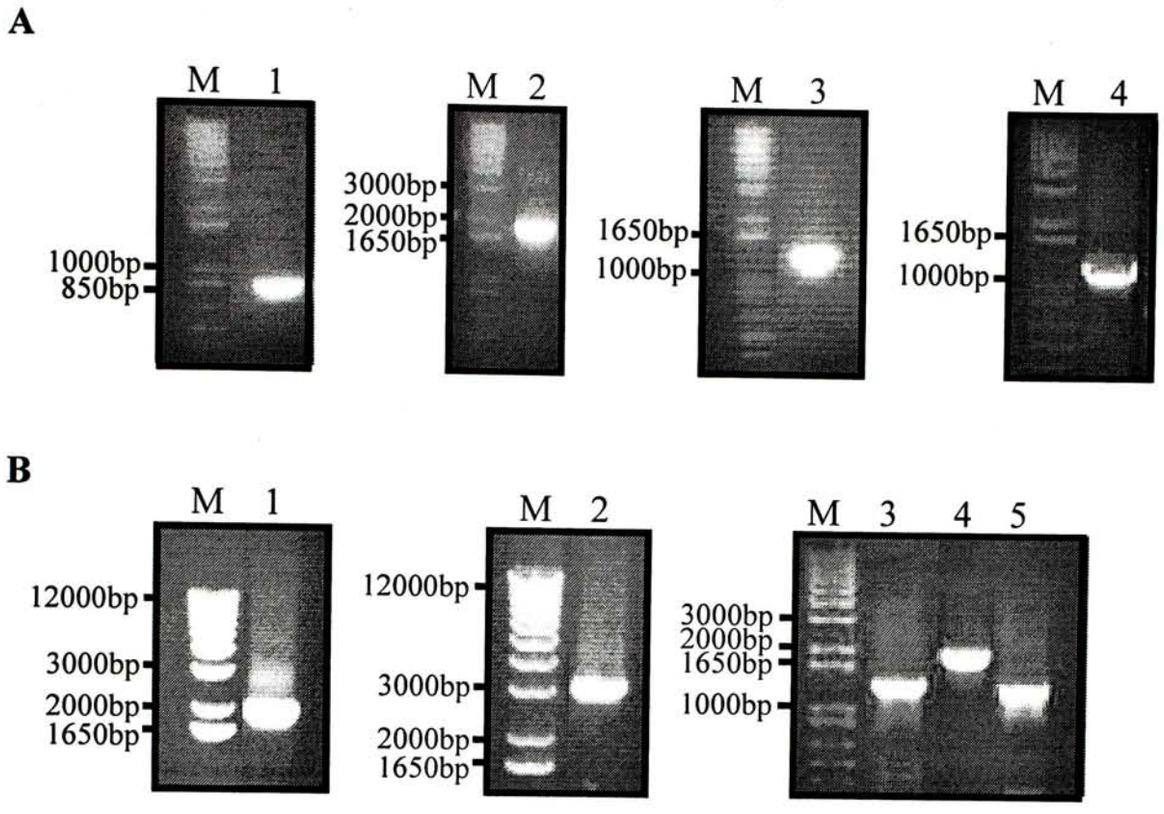


Figure 4.2 Construction of gene cassettes for *Ospsy1* and *Ospsy2*

(A) Cloning of required fragments: Genomic DNA was extracted from leaves of wild type *Japonica* 9983 rice and amplification of Gt-1_{pro} (1861bp) was carried out by PCR. pBI121 and J033025L21 plasmid DNAs were isolated and PCR was performed for the amplification of *CaMV35S*$_{pro}$ (801bp) and *Ospsy2* cDNA (1197bp) respectively. Total RNA was extracted from leaves of wild type *Japonica* 9983 rice and RT-PCR was performed for amplifying *Ospsy1* cDNA (1263bp). Products were analyzed by gel electrophoresis. Lanes: M, 1kb plus DNA marker, Invitrogen; 1, *CaMV35S*$_{pro}$; 2, Gt-1_{pro}; 3, *Ospsy1; and 4, Ospsy2.* **(B)** Confirmation of gene cassettes in pSB130 vector by PCR. pSB130 vectors with gene cassettes were isolated and PCR was carried out for confirmation. Products were analyzed by gel electrophoresis. Lanes: M, 1kb plus DNA marker, Invitrogen; 1, *35S*$_{pro}$*/Ospsy1* (2064bp); 2, Gt-1_{pro}*/Ospsy1* (3124bp); 3, *35S*$_{pro}$*/Ospsy2* (1998bp); 4 and 5, Gt-1_{pro} (1861bp) and *Ospsy2* (1197bp) from Gt-1_{pro}*/Ospsy2* respectively.

4.1.2 Rice transformation

Primary calli were induced using *Japonica* WYJ3 mature rice seeds which show better viral resistance than *Japonica* 9983 rice. The primary calli were co-cultivated with *Agrobacterium* harbouring the targeted constructs. The treated calli were washed and transferred onto selection medium. During the selection stage, the calli were placed in darkness for 2 to 3 months. The non-transformed calli turned dark and died while the transformed cells kept propagated to form resistant calli. Some of the resistant calli transformed with the $35S_{pro}$/*Ospsy1* and $35S_{pro}$/*Ospsy2* were orange, in contrast, those transformed with the empty vector, and the two Gt-1_{pro} driven constructs remained yellow, as depicted in Figure 4.3. Since a constitutive OsPSY expression was expected in the $35S_{pro}$ driven calli, and carotenoids are consisted of orange to red pigments, part of the orange calli were reserved for further analysis.

A total of 540 groups of resistant calli were subjected to pre-regeneration and regeneration treatment. However, due to the poor regeneration rate of *Japonica* WYJ3, only 113 individuals transgenic rice could be regenerated. The regenerated plants were planted in Gene Garden in March 2010.

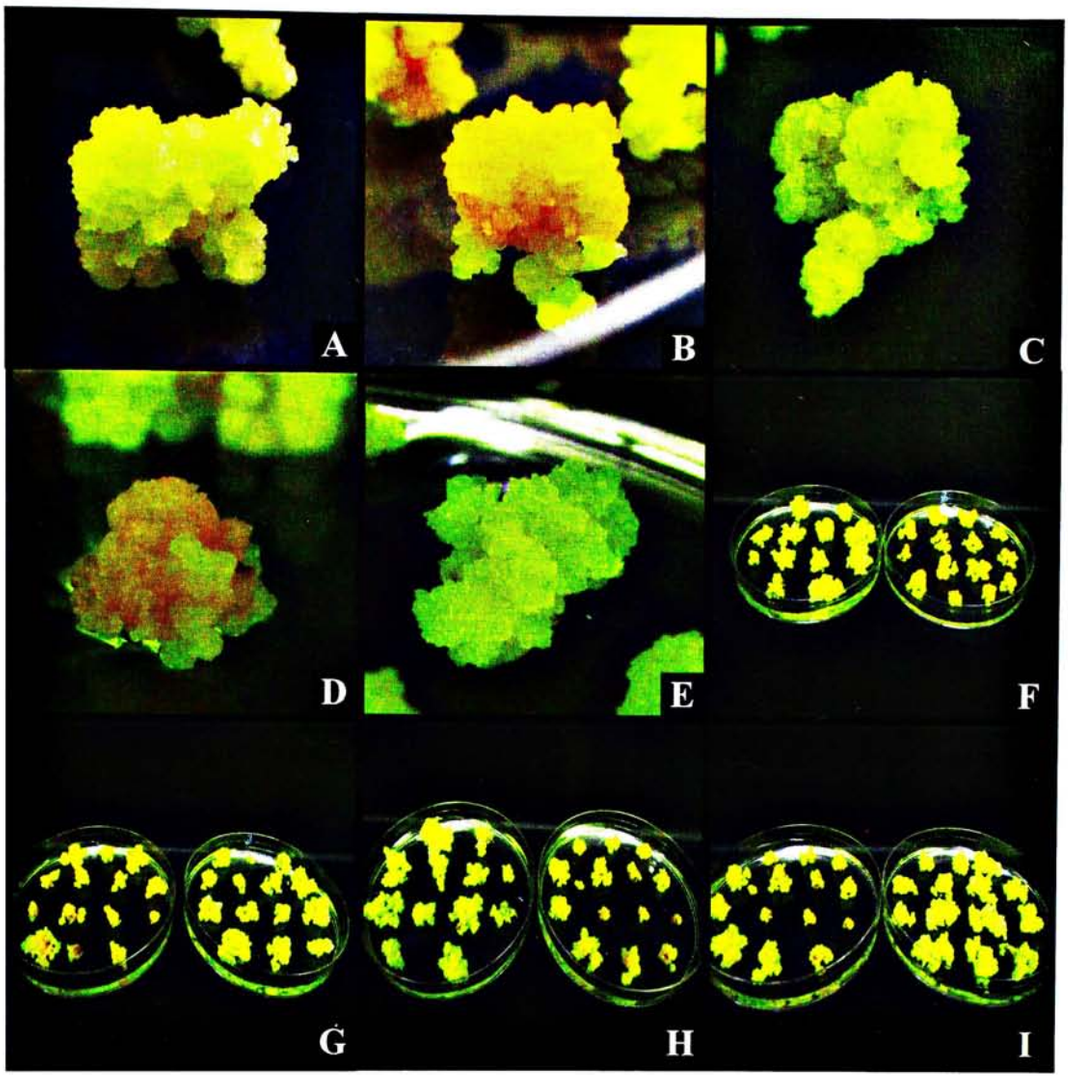


Figure 4.3 Resistant rice calli transformed with different *Ospsy* gene cassettes

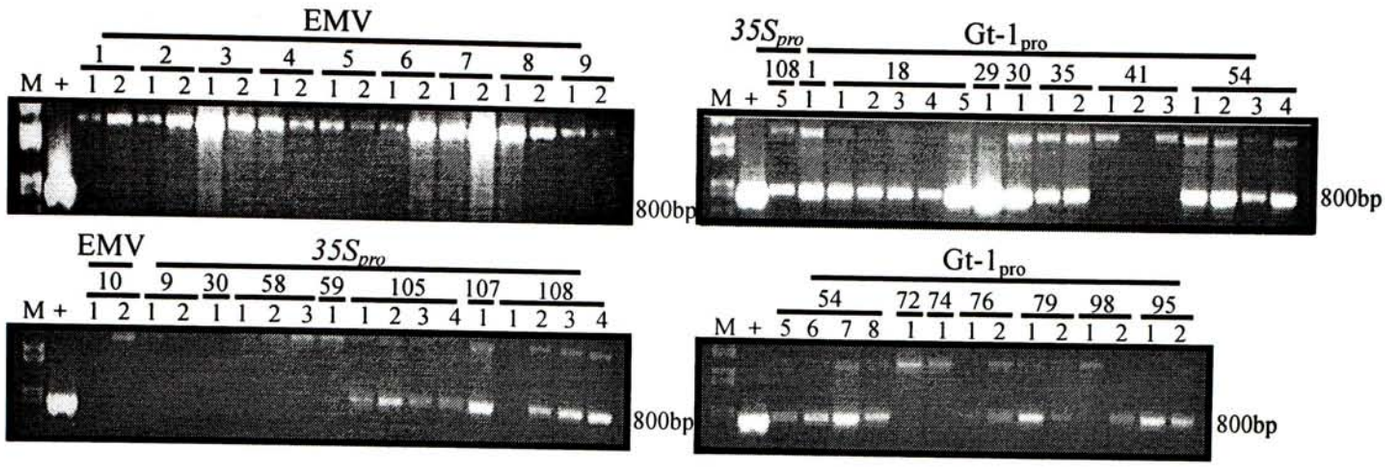
(A - E) Selected rice calli transformed with different gene cassettes. **(A)** Empty vector; **(B)** $35S_{pro}$/*Ospsy1*; **(C)** Gt-1_{pro}/*Ospsy1*; **(D)** $35S_{pro}$/*Ospsy2*; and **(E)** Gt-1_{pro}/*Ospsy2*. **(F - I)** Comparison of selected rice calli transformed with different gene cassettes. **(F)** Empty vector (Left) and $35S_{pro}$/*Ospsy1* (Right); **(G)** $35S_{pro}$/*Ospsy1* (Left) and Gt-1_{pro}/*Ospsy1* (Right); **(H)** Empty vector (Left) and $35S_{pro}$/*Ospsy2*(Right); and **(I)** $35S_{pro}$/*Ospsy2* (Left) and Gt-1_{pro}/*Ospsy2* (Right).

4.1.3 Transgene detection

4.1.3.1 Genomic DNA PCR screening

Genomic DNA was extracted from the leaves of all regenerated rice before plantation. PCR was carried out to screen for plants harboring the transgene. As the endogenous *Ospsy1* and *Ospsy2* in the rice genome could also give amplified products, in order to eliminate false-positive results, primer sets flanking the distal region of the 1st exon and the proximal region of the 4th exon were designed. PCR product from transgene was 800bp; in contrast, product from endogenous copy was about 3000bp. Genomic DNA PCR was preformed using primer sets P01 and P02 for *Ospsy1* constructs, and P03 and P04 for *Ospsy2* constructs. The PCR products were analyzed by gel electrophoresis as shown in Figure 4.4. All 20 empty vector plants and those with positive results were planted in Gene Garden.

OsPSY1



OsPSY2

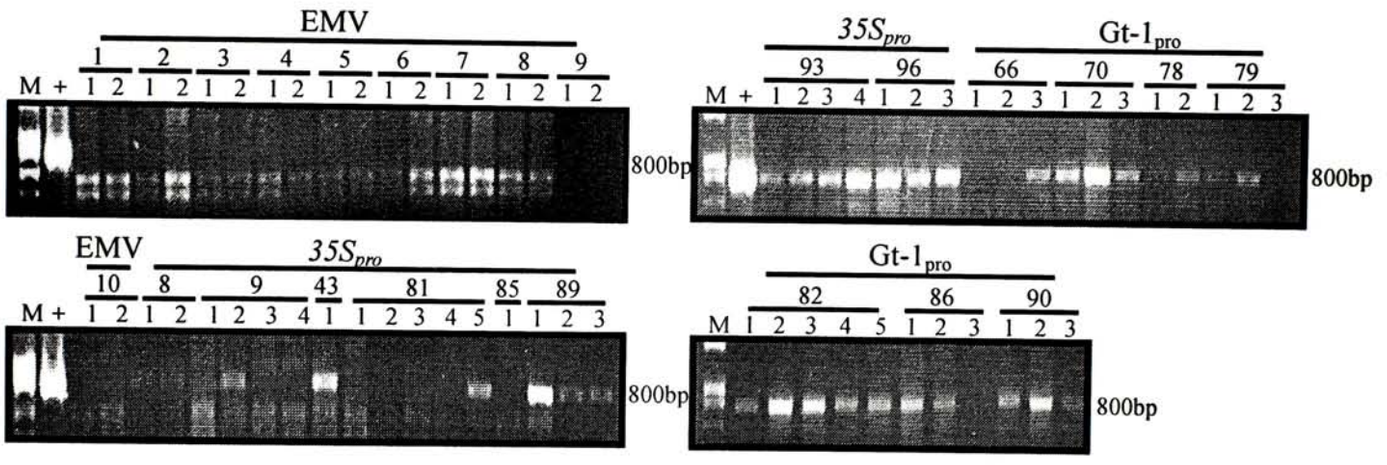


Figure 4.4 Genomic PCR of rice transformed with different *Ospsy* gene constructs

Genomic DNA was extracted from leaves of transgenic rice transformed with different *Ospsy* gene constructs. PCR was performed using primer sets P01 and P02, and, P03 and P04 for screening *Ospsy1* and *Ospsy2* plants respectively. Products were analyzed by gel electrophoresis. Expected size of *Ospsy* cDNA fragment was 800bp. Lanes: M, 1kb plus DNA marker, Invitrogen; and +, positive control using plasmid DNA of respective constructs.

4.1.3.2 Southern blot analysis

In order to determine the copy number of transgene in the genome of transgenic calli and regenerated plants, Southern blot analysis was performed. DIG-labeled double-stranded DNA probes specific of *Ospsy1* and *Ospsy2* were made by PCR using primer sets P01 and P02, and P03 and P04 respectively. The probes were subjected to dot blot analysis and their concentrations were determined by comparing the intensity of the probe signals with the known amount control DNA, as shown in Figure 4.5. For both *Ospsy1* and *Ospsy2* probes, their concentrations were 100ng/μl and their signals were strong enough to be used in Southern blot analysis.

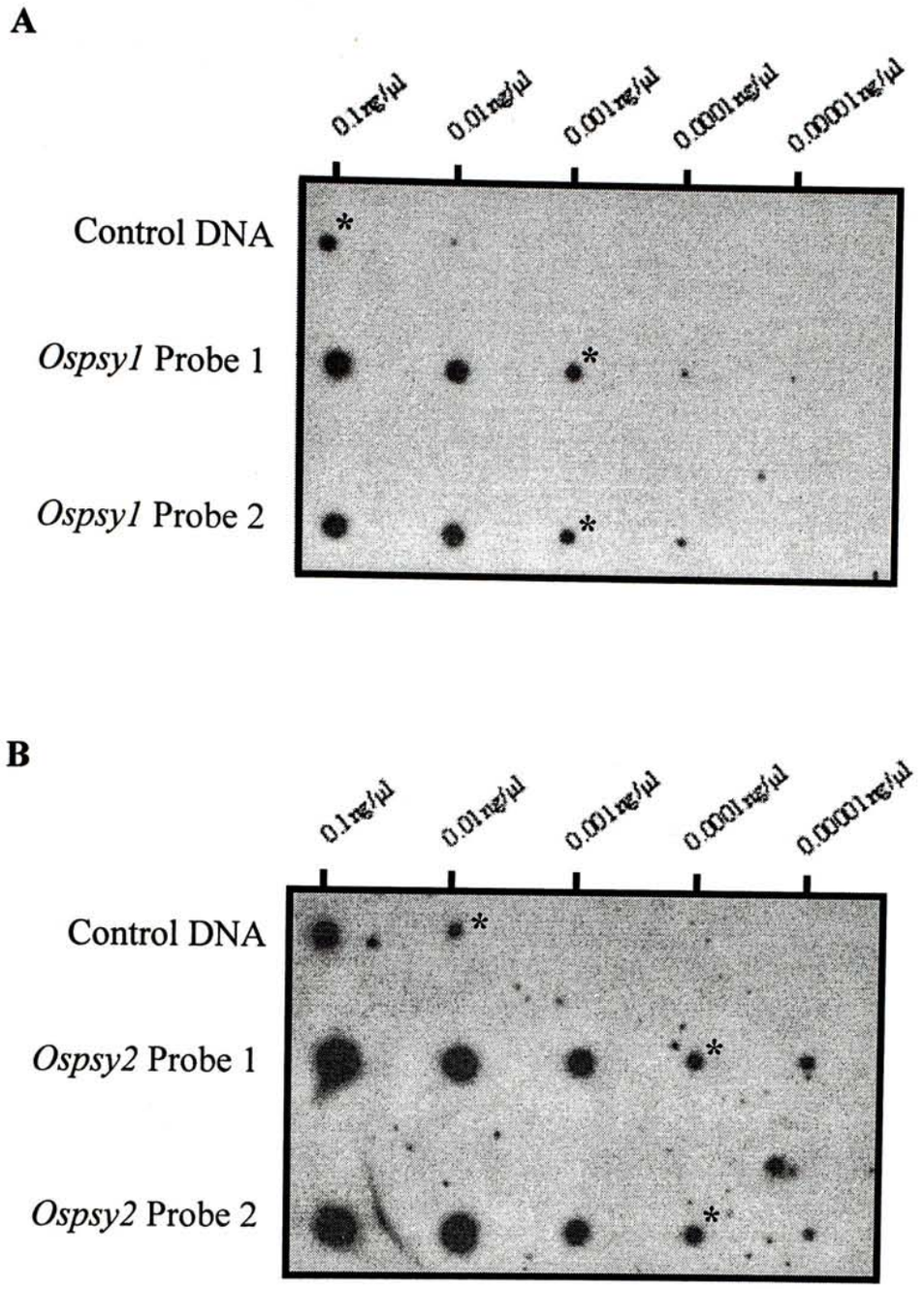


Figure 4.5 Dot blot analysis of DIG-labeled *Ospsy1* and *Ospsy2* DNA probes

DIG-labeled *Ospsy1* and *Ospsy2* DNA probes diluted to different concentrations were dotted on nylon membrane and detected with DIG Nucleic Acid Detection Kit (Roche Applied Science). **(A)** Dot blot analysis of *Ospsy1* double-stranded DNA probe; and **(B)** *Ospsy2* double-stranded DNA probe respectively. Compared with known amount of control DNA, the concentration of both the *Ospsy1* and *Ospsy2* DNA probes were 100ng/µl. Control DNA with intensity similar to that of DNA probes was marked with *.

4.1.3.2.1 Southern blot analysis on transgenic rice calli

In order to determine the presence of transgene in the genome of coloured calli, genomic DNA was extracted from calli of EMV, *35S$_{pro}$/Ospsy1*, Gt-1$_{pro}$/*Ospsy1*, *35S$_{pro}$/Ospsy2* and Gt-1$_{pro}$/*Ospsy2*. Southern blot analysis was performed. Genomic DNA samples were digested with BamHI overnight and the digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection of transgene was carried out using 500ng *Ospsy1* Probe 1 and *Ospsy2* Probe 2 for *Ospsy1* and *Ospsy2* calli respectively. Southern blot results were shown in Figure 4.6 which confirmed the presence of at least 1 copy of *35S$_{pro}$* driven *Ospsy* in the genome of the orange calli. As for the yellow calli with Gt-1$_{pro}$ driven *Ospsy*, at least 3 copies of transgene were detected in their genome. These confirmed the presence of gene cassettes in the respective calli.

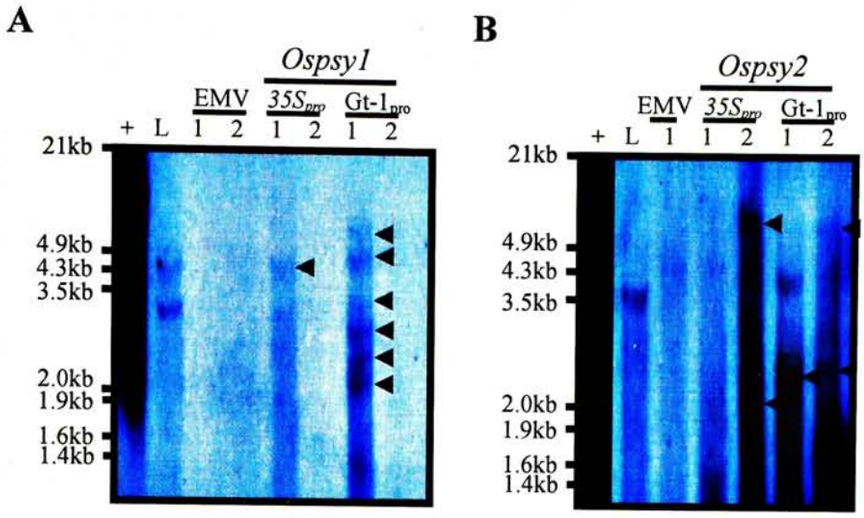


Figure 4.6 Southern blot analysis of transformed *Japonica* WYJ3 rice calli

Genomic DNA extracted from transgenic rice calli was digested with BamHI for empty vector, $35S_{pro}$/*Ospsy1*, Gt-1_{pro}/*Ospsy1*, $35S_{pro}$/*Ospsy2* and Gt-1_{pro}/*Ospsy2*. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using *Ospsy1* Probe 1 **(A)** and *Ospsy2* Probe 2 **(B)** respectively. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; and L, genomic DNA extracted from wild type WYJ3 rice leaves. The Southern blot results confirmed the presence of gene cassettes in the transgenic calli.

4.1.3.2.2 Southern blot analysis on regenerated rice

A month after plantation, rice leaf samples were collected from all regenerated rice and genomic DNA was extracted. Southern blot analysis was performed to determine the copy number of transgene in the genome of every individual. Genomic DNA was extracted from leaves of EMV, *35S$_{pro}$/Ospsy1*, Gt-1$_{pro}$/*Ospsy1*, *35S$_{pro}$/Ospsy2* and Gt-1$_{pro}$/*Ospsy2* plants. The DNA was subjected to the same treatment as the calli genomic DNA. The Southern blot results for the leaves samples were shown in Figure 4.7 and 4.8.

Sixty three individuals, excluding the EMV plants, showed positive results and generally contained one to two copies of transgene. The *35S$_{pro}$/Ospsy1* construct gave 10 positive plants from 6 individual lines of which 6 regenerated plants contained single copy and 4 contained two copies. The Gt-1$_{pro}$/*Ospsy1* construct formed 18 positive plants from 9 individual lines of which 14 plants contained singled copy, 2 with two copies and 2 with 4 copies. The *35S$_{pro}$/Ospsy2* construct gave 16 positive plants from 7 lines, and all of them contained single copy of transgene. Finally, the Gt-1$_{pro}$/*Ospsy2* construct yielded 19 positive plants from 7 lines of which 9 plants had single copy and 10 with two copies. Details of transgenic rice regeneration and Southern blot analysis were summarized in Table 4.1.

Ospsy1

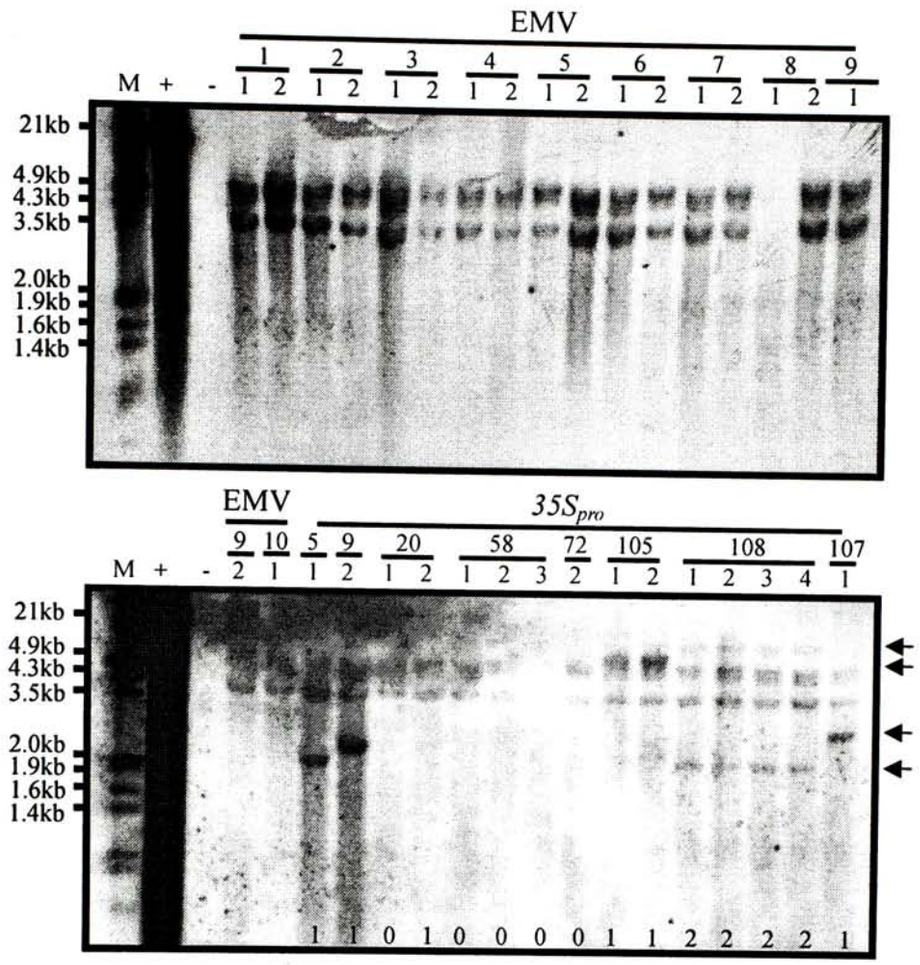


Figure 4.7 Southern blot analysis of *Ospsy1* transformed *Japonica WYJ3* rice

Genomic DNA extracted from transgenic rice leaves was digested with BamHI for empty vector, $35S_{pro}$/*Ospsy1* and Gt-1_{pro}/*Ospsy1*. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using *Ospsy1* Probe 1. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; and -, negative control. Due to the presence of endogenous *Ospsy* copy, signals were detected in EMV samples. Therefore, only bands additional to the signals given by the EMV were considered as positive.

Ospsy1

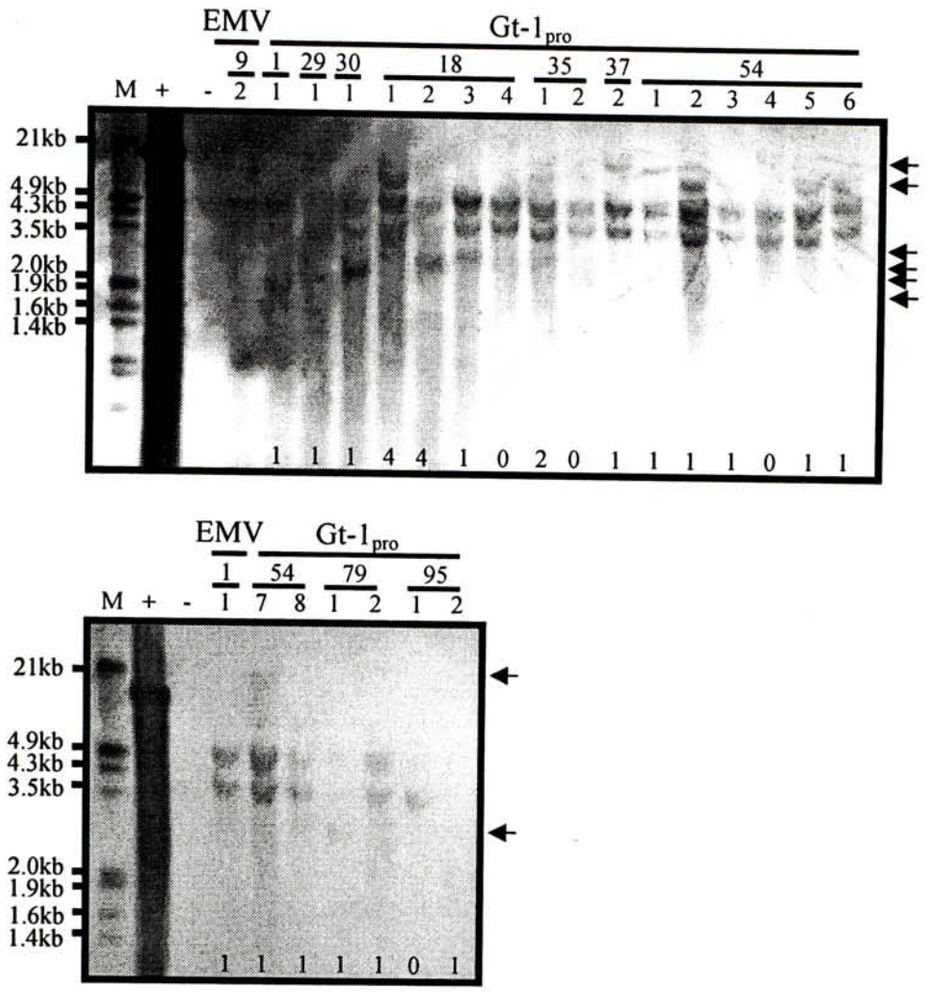


Figure 4.7 Southern blot analysis of *Ospsy1* transformed *Japonica WYJ3* rice (Continued)

Genomic DNA extracted from transgenic rice leaves was digested with BamHI for empty vector, *35S*$_{pro}$*/Ospsy1* and Gt-1$_{pro}$*/Ospsy1*. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using *Ospsy1* Probe 1. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; and -, negative control. Due to the presence of endogenous *Ospsy* copy, signals were detected in EMV samples. Therefore, only bands additional to the signals given by the EMV were considered as positive.

Ospsy2

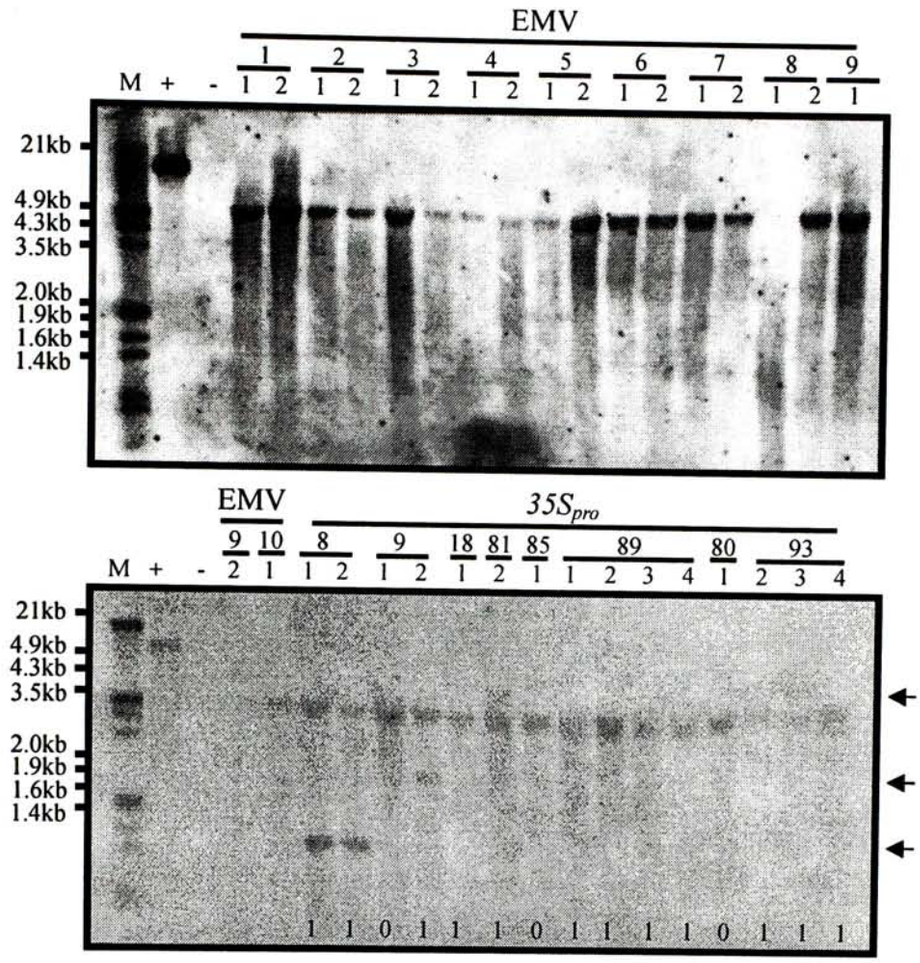


Figure 4.8 Southern blot analysis of *Ospsy2* transformed *Japonica WYJ3* rice

Genomic DNA extracted from transgenic rice leaves was digested with BamHI for empty vector, $35S_{pro}$/*Ospsy2* and Gt-1_{pro}/*Ospsy2*. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using *Ospsy2* Probe 2. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; and -, negative control. Due to the presence of endogenous *Ospsy* copy, signals were detected in EMV samples. Therefore, only bands additional to the signals given by the EMV were considered as positive.

Ospsy2

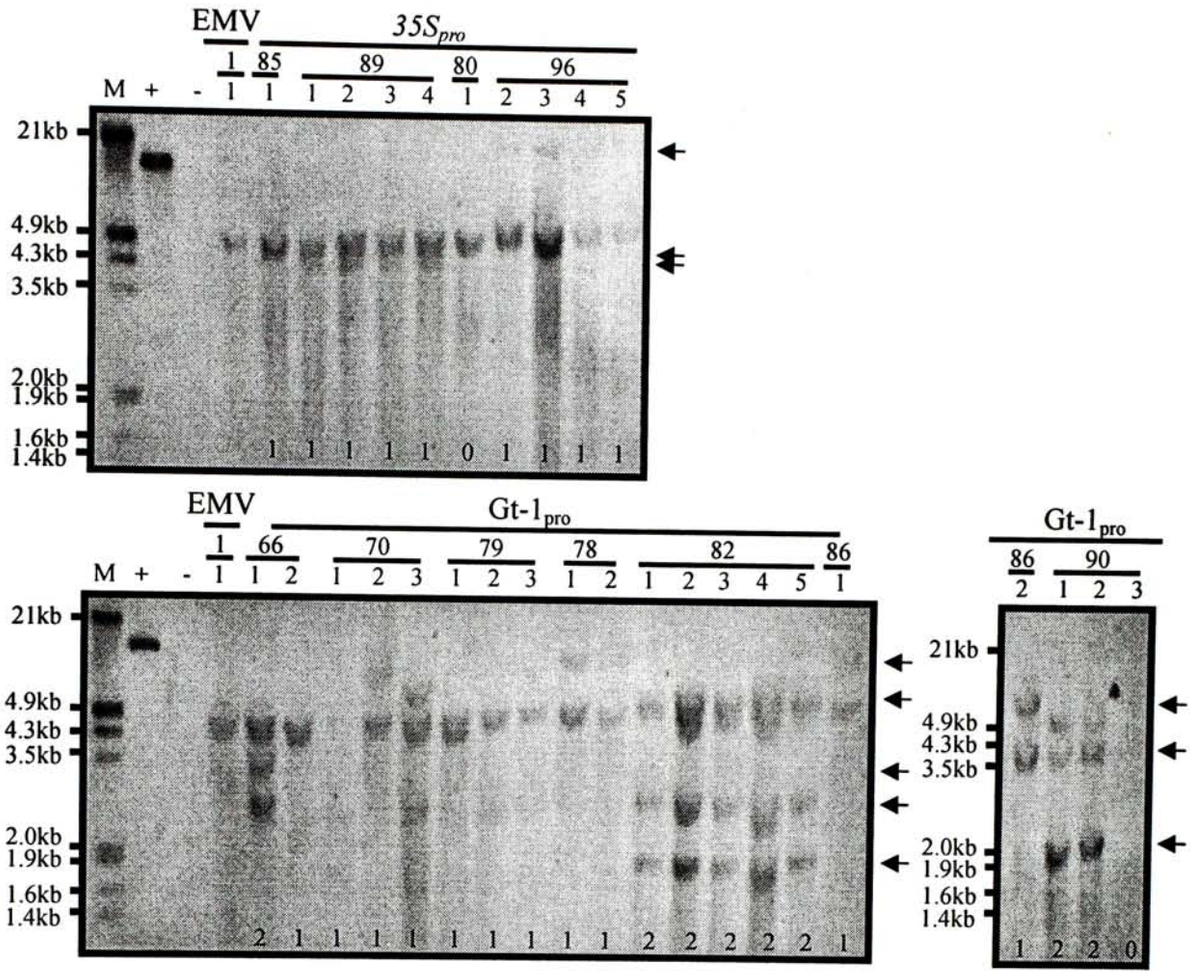


Figure 4.8 Southern blot analysis of *Ospsy2* transformed *Japonica WYJ3* rice (Continued)

Genomic DNA extracted from transgenic rice leaves was digested with BamHI for empty vector, *35S*$_{pro}$*/Ospsy2* and Gt-1$_{pro}$*/Ospsy2*. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using *Ospsy2* Probe 2. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; and -, negative control. Due to the presence of endogenous *Ospsy* copy, signals were detected in EMV samples. Therefore, only bands additional to the signals given by the EMV were considered as positive.

Table 4.1 Summary of regeneration of transgenic rice and copy number of transgene of different gene constructs

Constructs	Resistant calli	Regenerated tarnsgenic plants			Copy number of transgene			
		Number of line	Number of individual plants	Number of positive transformants planted (lines)	1	2	3	4
Empty vector	80	10	20	20 (10)	0	0	0	0
pSB130/$35S_{pro}$/*Ospsy1*	120	7	17	10 (6)	6	4	0	0
pSB130/Gt-1_{pro}*Ospsy1*	110	13	31	18 (9)	14	2	0	2
pSB130/$35S_{pro}$/*Ospsy2*	120	8	23	16 (7)	16	0	0	0
pSB130/Gt-1_{pro}*Ospsy2*	110	7	22	19 (7)	9	10	0	0
Total number of plants grown				83				

4.1.4 Detection of transgene expression

4.1.4.1 Northern blot analysis on immature transgenic seed

Immature seeds (20 DAF) were collected from 58 transgenic plants with positive results in Southern blot analysis. Total RNA was extracted and resolved in 1% agarose/formaldehyde gel. Then, the RNA samples were transferred onto nylon membrane and detection was carried out using *Ospsy1* Probe 1 and *Ospsy2* Probe 2 for *Ospsy1* and *Ospsy2* constructs respectively. Total RNA extracted from EMV immature seed was used as a negative control. The northern blot results were illustrated in Figures 4.9 and 4.10. In the $35S_{pro}$/*Ospsy1* samples, 3 out of 12 showed expression of *Ospsy1* in the immature endosperm while 8 out of 17 were detected for Gt-1_{pro}/*Ospsy1*. As for *Ospsy2*, 10 out of 13 of the $35S_{pro}$ driven samples showed increased *Ospsy2* transcript level while 15 out of 16 were detected for Gt-1_{pro} driven samples.

OsPSY1

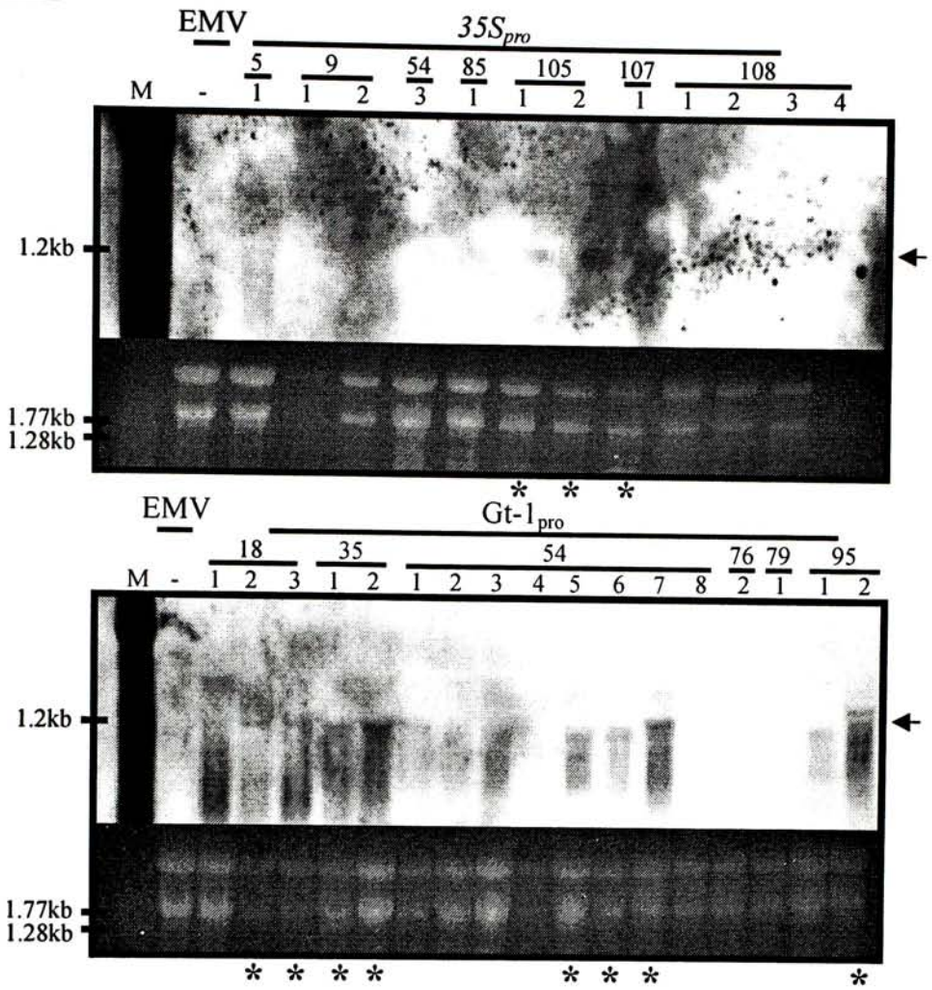


Figure 4.9 Northern blot analysis of *Ospsy1* transformed *Japonica* WYJ3 rice immature seeds

Total RNA extracted from immature transgenic rice seeds of empty vector, *35S$_{pro}$/Ospsy1*, and Gt-1$_{pro}$/*Ospsy1* were resolved in 1% agarose/formaldehyde gel and transferred onto nylon membrane. Detection was carried out using *Ospsy1* Probe 1. Positive signals are indicated by arrows. Lanes: M, 0.16 - 1.77kb RNA ladder, Invitrogen; -, negative control. Individuals with positive signals were marked with *.

OsPSY2

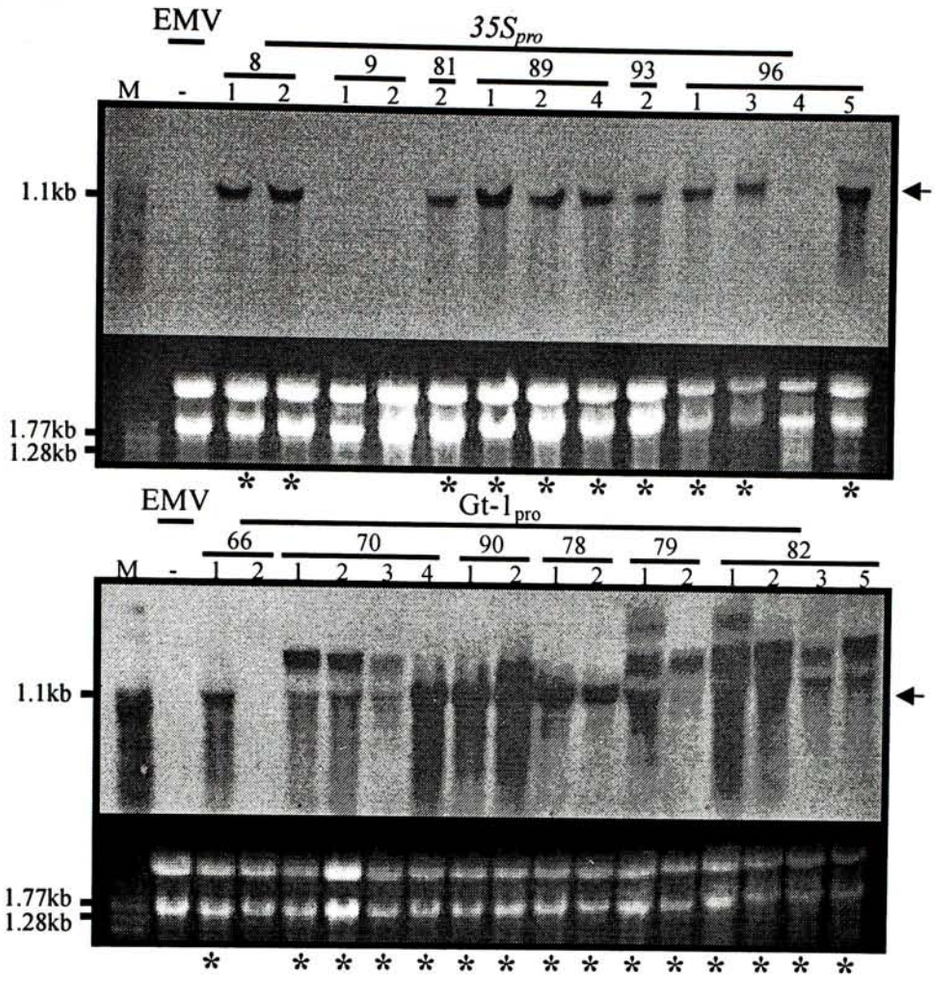


Figure 4.10 Northern blot analysis of *Ospsy2* transformed *Japonica* WYJ3 rice immature seeds

Total RNA extracted from immature transgenic rice seeds of empty vector, *35S$_{pro}$/Ospsy2*, and Gt-1$_{pro}$/*Ospsy2* were resolved in 1% agarose/formaldehyde gel and transferred onto nylon membrane. Detection was carried out using *Ospsy*2 Probe 2. Positive signals are indicated by arrows. Lanes: M, 0.16 - 1.77kb RNA ladder, Invitrogen; -, negative control. Individuals with positive signals were marked with *.

4.1.4.2 Western blot analysis on transgenic rice tissues

4.1.4.2.1 Antibody production

The cDNAs of *Ospsy1* and *Ospsy2* were cloned into pET30a vector for protein induction with the bacterial expression system. The sequences were verified by DNA sequencing to ensure correct sequence and no frame shift was detected. The vectors with correct cDNAs were used to transform *E.coli* Rosetta (2) for protein expression. After IPTG induction, an extra protein band was observed in the proteins of bacteria transformed with *Ospsy1* and *Ospsy2* respectively. The bands shown expected sizes of the induced 52kDa OsPSY1 and 50kDa OsPSY2. The total bacterial protein extract was resolved by tricine-SDS PAGE, and the extra protein bands were collected. Gel elution was performed for three times to purify the induced OsPSY proteins. Quantitation of the purified proteins was carried out by comparing to known amount of BSA standard, as shown in Figure 4.11. The estimated concentrations of the induced OsPSY1 and OsPSY2 were both at 6μg/10μl.

A total of 600μg of each induced OsPSY proteins was used to immunize rabbits for antibody production in four separate injections. Sera from immunized rabbits were collected and the titer was checked by dot blot analysis as presented in Figure 4.12.

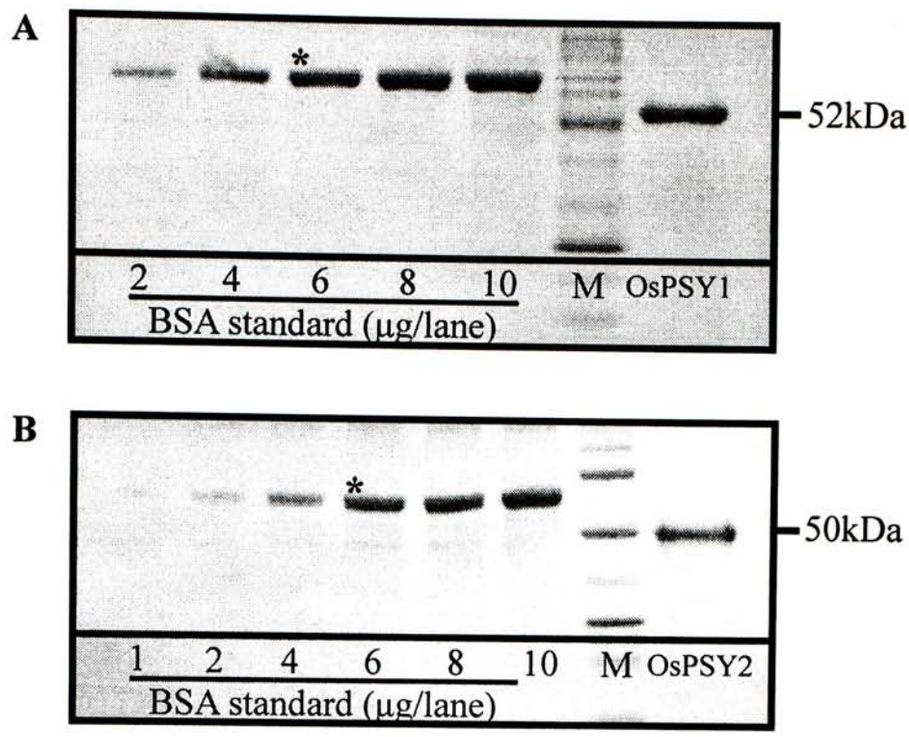


Figure 4.11 Purified OsPSY1 and OsPSY2 proteins compared with BSA standard

OsPSY1 and OsPSY2 proteins induced with the bacterial system were gel purified and quantitated by comparing with known amounts of BSA standard. **(A)** Purified OsPSY1 protein and **(B)** purified OsPSY2 protein were both about 6ng/lane after 3rd round of purification. Lanes: 1, 2, 4, 6, 8, 10, amount of BSA standard in microgram loaded into each lane; M, BenchMark Protein Ladder, (Invitrogen), OsPSY1 and OsPSY2, bacterial induced protein purified after three rounds of gel purification. BSA standard with intensity similar to that of the purified OsPSY protein was marked with *.

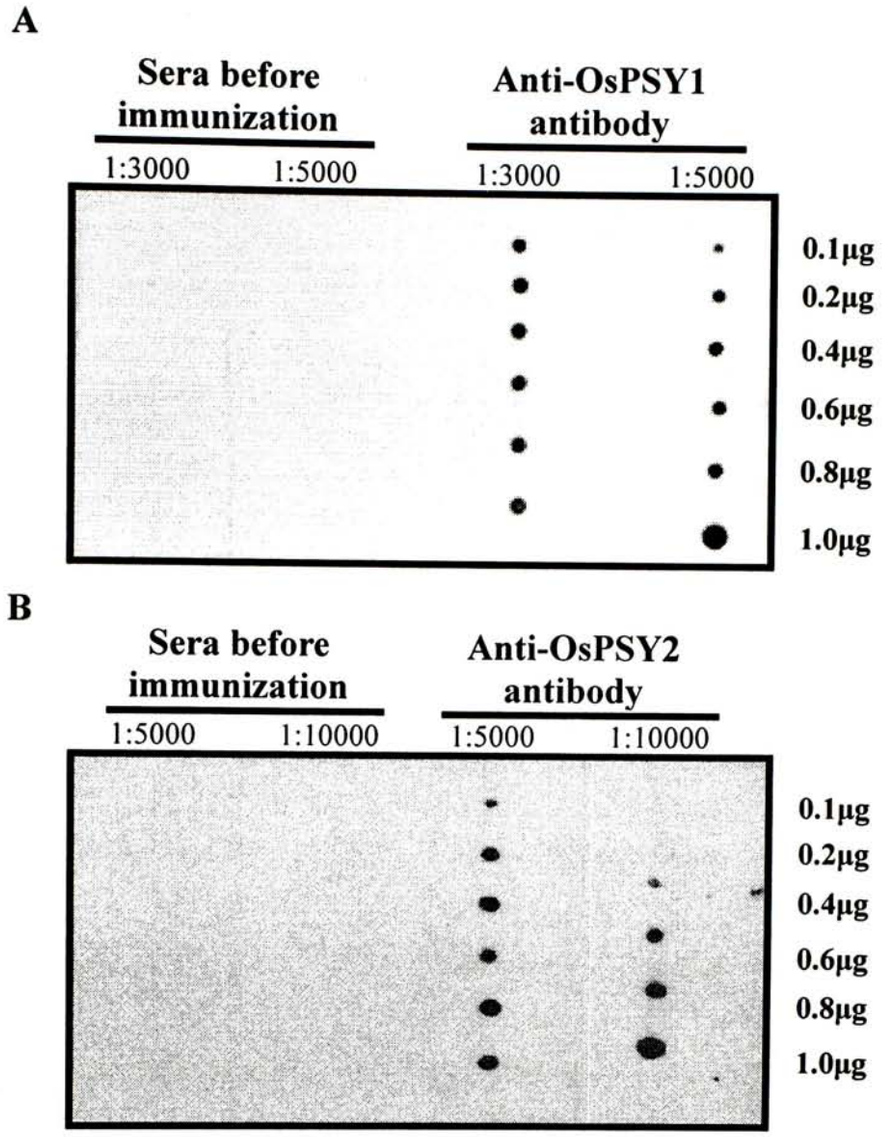


Figure 4.12 Dot blot analysis of anti-OsPSY1 and anti-OsPSY2 sera titer

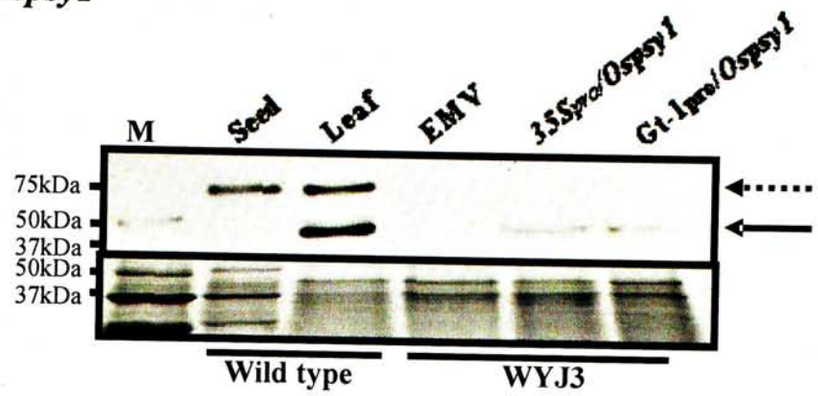
Bacterial induced **(A)** OsPSY1 and **(B)** OsPSY2 proteins with different concentrations were dotted onto nitrocellulose membrane and reacted with different dilutions of antisera respectively. **(A)** OsPSY1 antigen was dotted onto the membrane. Rabbit sera collected before immunization and anti-OsPSY1 antibody with dilution of 1:3000 and 1:5000 were used for detection; and **(B)** OsPSY2 antigen was dotted onto the membrane. Rabbit sera collected before immunization and anti-OsPSY2 antibody with dilution of 1:5000 and 1:10000 were used for detection.

4.1.4.2.2 Western blot analysis of transgenic rice calli

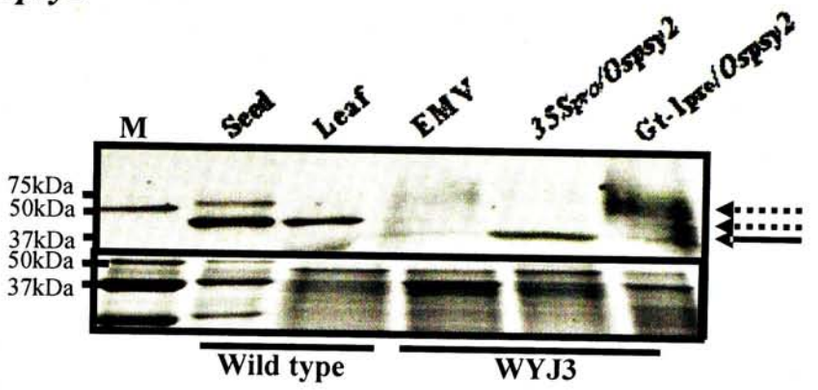
In order to determine the OsPSYs expression in the orange calli, western blot analysis was carried out. Total protein was extracted from freeze-dried calli of EMV, *35S$_{pro}$/Ospsy1*, Gt-1$_{pro}$/*Ospsy1*, *35S$_{pro}$/Ospsy2* and Gt-1$_{pro}$/*Ospsy2*. After resolution by tricine-SDS PAGE, the proteins were transferred onto PVDF membranes. Detection was carried out using anti-OsPSY1 and anti-OsPSY2 antibodies at a dilution of 1:3000 for *Ospsy1* and *Ospsy2* calli respectively. Western blot results were illustrated in Figure 4.13(A) and (B).

The strongest signals of OsPSY1 and OsPSY2 were detected in the orange *35S$_{pro}$/Ospsy1* and *35S$_{pro}$/Ospsy2* calli respectively. This confirmed the expression of transgenes in the orange transgenic calli. As a weak expression of OsPSY1 and OsPSY2 was also detected in the two yellow calli with *Ospsy* genes driven by the seed-specific Gt-1$_{pro}$, therefore, a Gt-1$_{pro}$/GUS construct was transformed into WYJ3 rice to reveal the Gt-1$_{pro}$ activity in calli. Histochemical staining of GUS of the transgenic calli proved that the seed-specific Gt-1$_{pro}$ could direct a weaker GUS expression in rice calli when compared with *35S$_{pro}$*, (Figure 4.13 C). Finally, a very weak OsPSY2 expression was also detected in EMV calli, (Figure 4.13 B), which may be due to the expression of the endogenous copy. In summary, the western blot results suggested a higher level of transgene expression in the orange transgenic calli.

A. *Ospsy1*



B. *Ospsy2*



C.

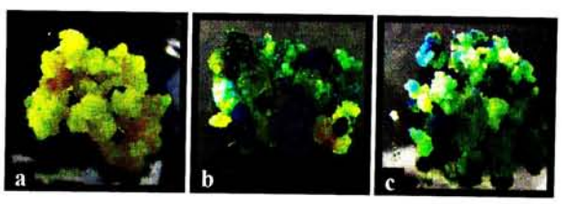


Figure 4.13 Western blot analysis of *Ospsy1 and Ospsy2* transgenic expression in *Japonica* WYJ3 rice calli

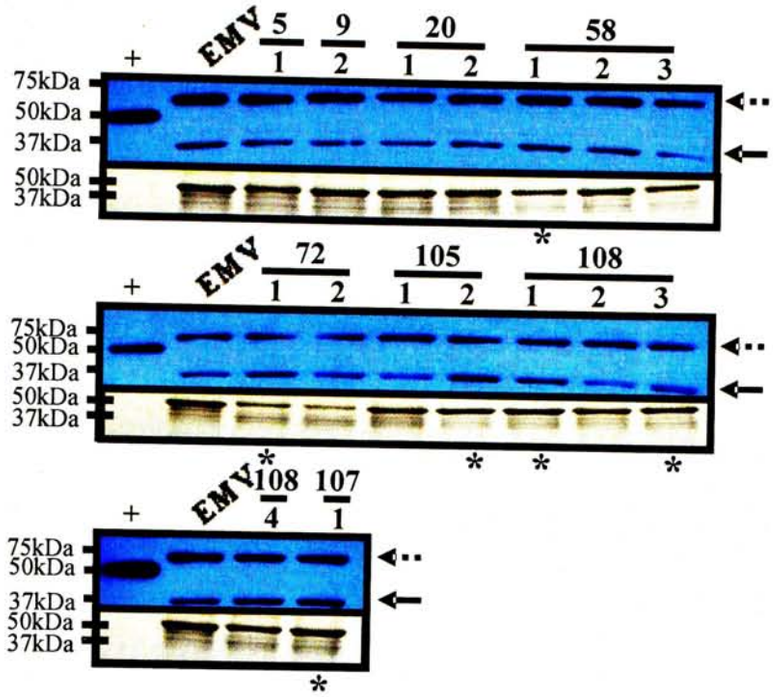
Total protein was extracted from transgenic rice calli transformed with different *Ospsy* gene cassettes and was resolved by tricine SDS-PAGE. After transferring onto PVDF membrane, detection was carried out using anti-OsPSY1 and anti-OsPSY2 antibodies. **(A)** Western blot (upper) and Commassie Blue stain (lower) of *Ospsy1* calli using 1:3000 anti-OsPSY1 antibody and **(B)** western blot (upper) and Commassie Blue stain (lower) of *Ospsy2* calli using 1:3000 anti-OsPSY2 antibodies. Lanes: M, Precision Plus Protein Dual Color Standards (Bio-Rad); Seed, total protein extracted from mature wild type WYJ3 seed; Leaf, total protein extracted from wild type WYJ3 leaf; EMV, total protein extracted from rice calli transformed with empty pSB130 vector; $35S_{pro}$/*Ospsy1*, total protein from rice calli with $35S_{pro}$/*Ospsy1* cassette; Gt-1_{pro}/*Ospsy1*, total protein from rice calli with Gt-1_{pro}/*Ospsy1*; $35S_{pro}$/*Ospsy2*, total protein from rice calli with $35S_{pro}$/*Ospsy2*cassette; and Gt-1_{pro}/*Ospsy2*, total protein from rice calli with Gt-1_{pro}/*Ospsy2*. Bands with expected size of target proteins indicated with solid arrows; while unidentified bands indicated with dotted arrows. **(C)** Histochemical staining of GUS in WYJ3 calli transformed with **(a)** empty vector, **(b)** $35S_{pro}$/GUS, and **(c)** Gt-1_{pro}/GUS cassettes.

4.1.4.2.3 Western blot analysis of transgenic rice leaves

Total protein was extracted from fresh leaves of EMV, *35S*$_{pro}$*/Ospsy1*, Gt-1$_{pro}$*/Ospsy1*, *35S*$_{pro}$*/Ospsy2* and Gt-1$_{pro}$*/Ospsy2*. After resolution by tricine-SDS PAGE, the protein samples were transferred onto PVDF membranes. Detection was carried out using anti-OsPSY1 and anti-OsPSY2 antibodies at a dilution of 1:3000 for *Ospsy1* and *Ospsy2* respectively. Western blot results of *Ospsy1* and *Ospsy2* leaves were illustrated in Figures 4.14 and 4.15.

As there were expressions of endogenous OsPSY1 and OsPSY2 in rice leaves, the expression of transgene should reveal a stronger signal when compared with the EMV. However, the increase in signal intensity was not obvious. Among the 78 samples tested, only 6 from *35S*$_{pro}$*/Ospsy1*, 6 from Gt-1$_{pro}$*/Ospsy1*, 9 from *35S*$_{pro}$*/Ospsy2* and 8 from Gt-1$_{pro}$*/Ospsy2* showed increased OsPSYs expression. The unobvious increase of transgene expression may be due to the expression of the endogenous copies was too high, making it difficult to detect the expression of the transgene.

A. *35S*$_{pro}$



B. Gt-1$_{pro}$

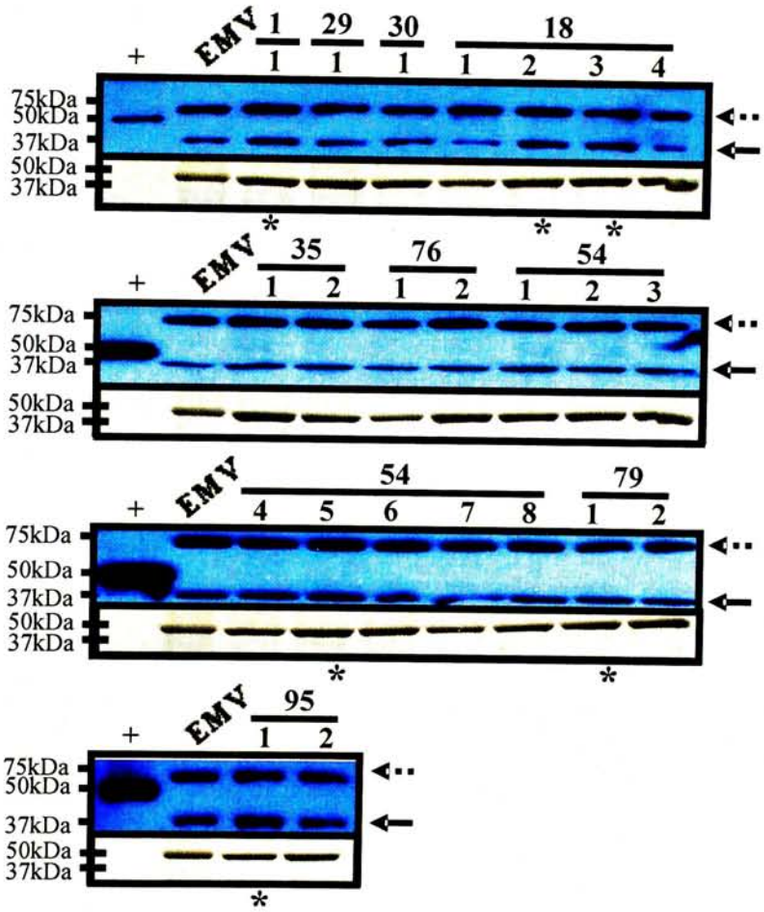
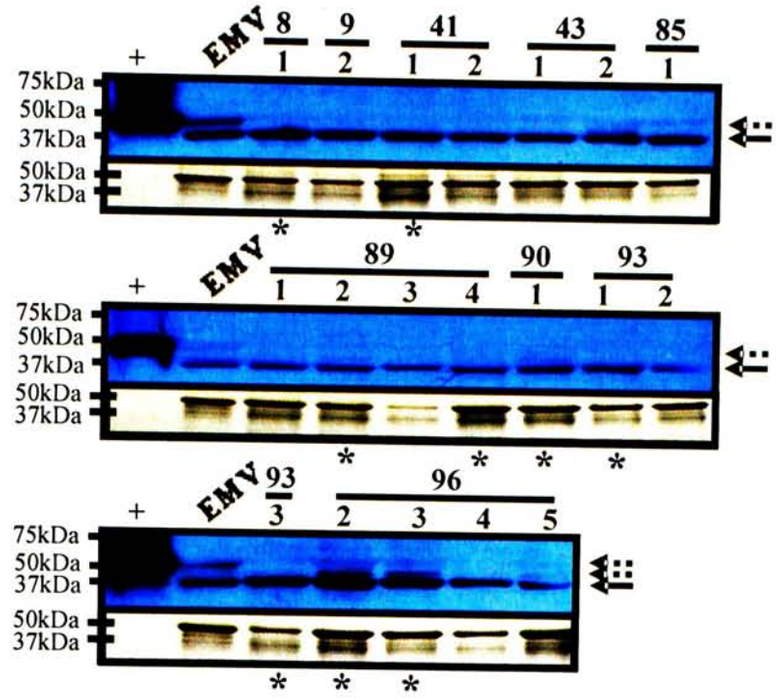


Figure 4.14 Western blot analysis of *Ospsy1* transgenic rice leaves

Total protein was extracted from transgenic rice leaves transformed with *Ospsy1* gene cassettes (A. *35S*$_{pro}$ and B. Gt-1$_{pro}$) and was resolved by tricine SDS-PAGE. After transferring onto PVDF membrane, detection was carried out using 1:3000 anti-OsPSY1antibody. Lanes: +, positive control of induced OsPSY1 protein; and EMV, total protein extracted from rice leaves transformed with empty pSB130 vector, upper number refers to transgenic line and lower number refers to individual plant in each line. Bands with expected size of *Ospsy1* proteins were indicated by solid arrows while unidentified bands indicated by dotted arrows. Band signals stronger than EMV were marked with *.

A. *35S*$_{pro}$



B. Gt-1$_{pro}$

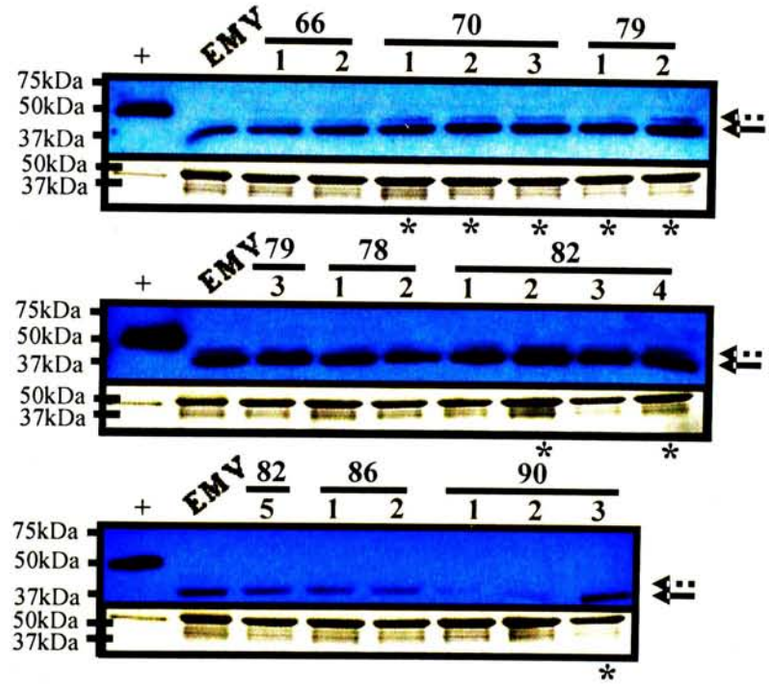


Figure 4.15 Western blot analysis of *Ospsy2* transgenic rice leaves

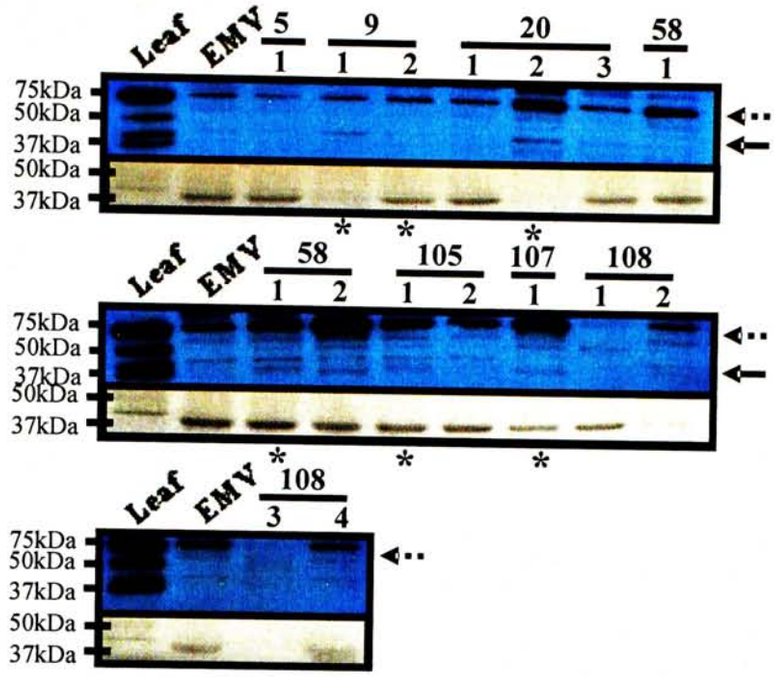
Total protein was extracted from transgenic rice leaves transformed with *Ospsy2* gene cassettes (A. *35S*$_{pro}$ and B. Gt-1$_{pro}$) and was resolved by tricine SDS-PAGE. After transferring onto PVDF membrane, detection was carried out using 1:3000 anti-OsPSY2antibody. Lanes: +, positive control of induced OsPSY2 protein; and EMV, total protein extracted from rice leaves transformed with empty pSB130 vector, upper number refers to transgenic line and lower number refers to individual plant in each line. Bands with expected size of *Ospsy2* proteins were indicated by solid arrows while unidentified bands indicated by dotted arrows. Band signals stronger than EMV were marked with *.

4.1.4.2.4 Western blot analysis of immature transgenic rice seeds

Total protein was extracted from dehusked immature seeds of EMV, *35S$_{pro}$/Ospsy1*, Gt-1$_{pro}$/*Ospsy1*, *35S$_{pro}$/Ospsy2* and Gt-1$_{pro}$/*Ospsy2*. After resolution by tricine-SDS PAGE, the protein samples were transferred onto PVDF membranes. Detection was carried out using anti-OsPSY1 and anti-OsPSY2 antibodies. Since the OsPSYs amount in seeds was low, antibodies used for detection was at a dilution of 1:1500 for both *Ospsy1* and *Ospsy2*. Western blot results of *Ospsy1* and *Ospsy2* immature seeds were illustrated in Figures 4.16, 4.17 (A) and 4.17 (B).

Western blot of *Ospsy1* immature seeds revealed that only 6 samples of *35S$_{pro}$/Ospsy1* and 6 samples of Gt-1$_{pro}$/*Ospsy1* showed expression of OsPSY1, which was not detected in the EMV sample. In contrast, similar amount of OsPSY2 was detected in all *Ospsy2* immature seeds and also for the EMV sample. This suggests an expression of endogenous OsPSY2 in the immature seeds. Thus, OsPSY2 expression level in different parts of EMV immature rice seed was determined, which revealed that the majority of OsPSY2 was expressed in the seed coat, as shown in Figure 4.17 (C).

A. $35S_{pro}$



B. Gt-1_{pro}

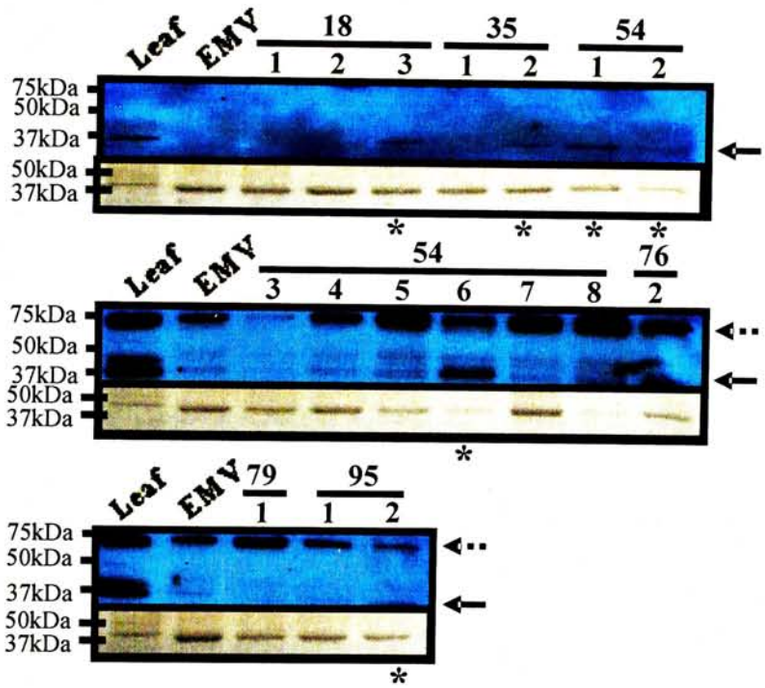


Figure 4.16 Western blot analysis of *Ospsy1* transgenic immature rice seeds

Total protein was extracted from transgenic immature rice seeds transformed with *Ospsy1* gene cassettes (A. $35S_{pro}$ and B. Gt-1_{pro}) and was resolved by tricine SDS-PAGE. After transferring onto PVDF membrane, detection was carried out using 1:1500 anti-OsPSY1antibody. Lanes: Leaf, total protein extracted from wild type WYJ3 leaves; and EMV, total protein extracted from immature rice seeds transformed with empty pSB130 vector, upper number refers to transgenic line and lower number refers to individual plant in each line. Bands with expected size of *Ospsy1* protein were indicated by solid arrows while unidentified bands were indicated by dotted arrows. Positive band signals were marked with *.

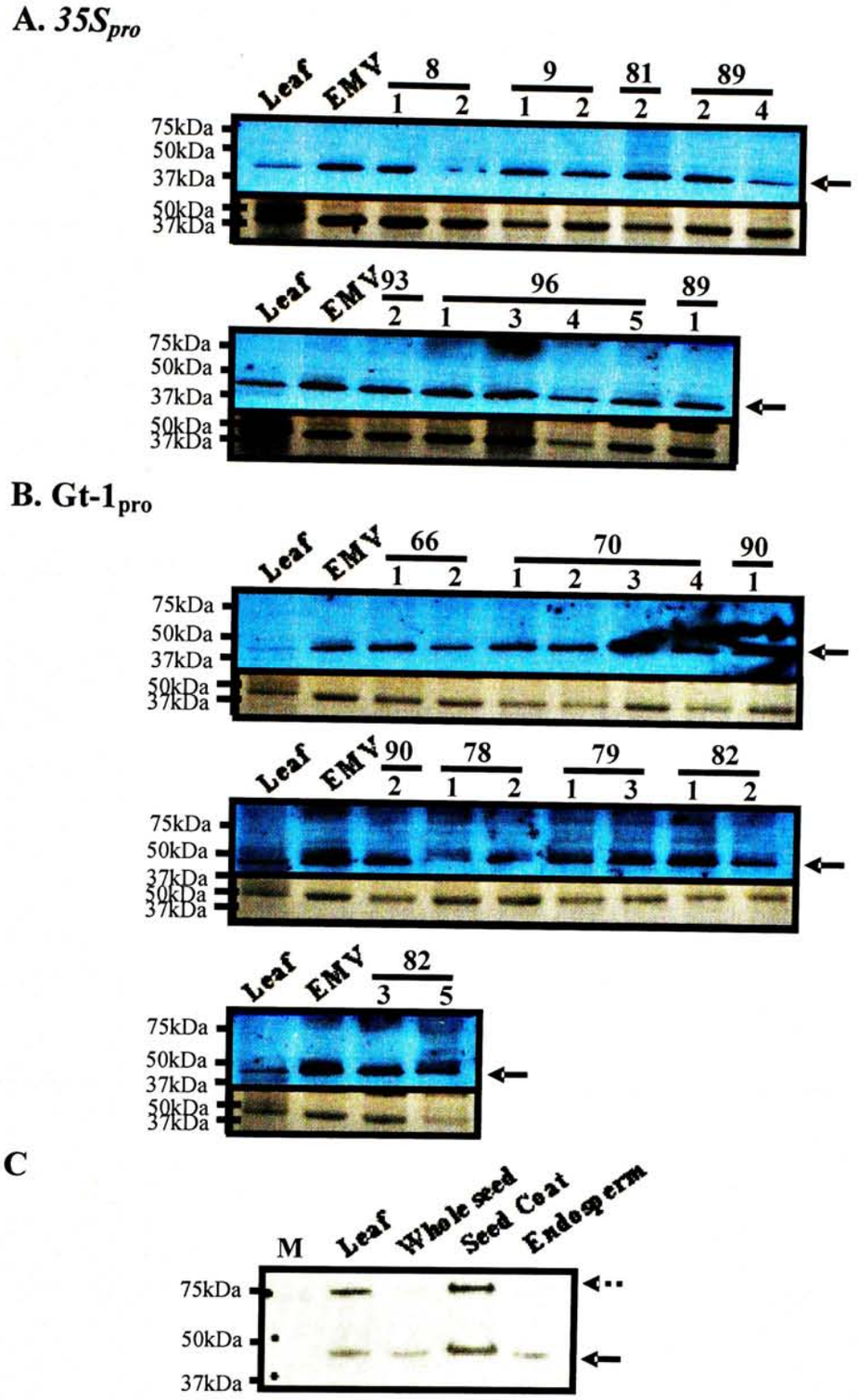


Figure 4.17 Western blot analysis of *Ospsy2* transgenic immature rice seeds

Total protein was extracted from transgenic immature rice seeds transformed with *Ospsy2* gene cassettes (A. $35S_{pro}$ and B. Gt-1_{pro}) and was resolved by tricine SDS-PAGE. After transferring onto PVDF membrane, detection was carried out using 1:1500 anti-OsPSY2antibody. Lanes: Leaf, total protein extracted from wild type WYJ3 leaves; and EMV, total protein extracted from immature rice seeds transformed with empty pSB130 vector, upper number refers to transgenic line and lower number refers to individual plant in each line. **(C)** Western blot of different parts of immature rice seed using 1:1500 anti-OsPSY2 antibody. Lanes: M, Precision Plus Protein Dual Color Standards, BioRad; Leaf, total protein extracted from wild type WYJ3 leaves; and total proteins extracted from whole grain, seed coat and endosperm of immature rice seeds. Bands with expected size of *Ospsy2* proteins were indicated by solid arrows while unidentified bands were indicated by dotted arrows.

4.1.5 Detection of OsPSYs activity at metabolite level

4.1.5.1 UPLC analysis of transgenic rice tissues

4.1.5.1.1 Carotenoid profiling of transgenic rice calli

Carotenoid profiles of 10 groups of rice calli from each gene construct were determined. Carotenoids in the freeze-dried transgenic rice calli were extracted and subjected to UPLC analysis. Phytoene was followed at 280nm while β-carotene at 477nm.

Phytoene was detected in all rice calli samples including the EMV, as indicated by the peak with an arrow in the chromatograms in Figure 4.18. This suggests the presence of endogenous OsPSY in all rice calli. As for those harboring the gene cassettes, an increase in phytoene level was observed for *35S$_{pro}$/Ospsy1*, *35S$_{pro}$/Ospsy2* and Gt-1$_{pro}$/*Ospsy2*, reaching 5042ng/mg, 3450ng/mg and 2418ng/mg of dry mass of calli (p <0.05). In contrast, phytoene level in Gt-1$_{pro}$/*Ospsy1* was 388ng/mg of dry mass of calli, which was lower than that of EMV. Since the p-value of Gt-1$_{pro}$/*Ospsy1* sample was greater than 0.05, there was no statistically significant difference in phytoene level between Gt-1$_{pro}$/*Ospsy1* sample and EMV. Among the four gene constructs, the use of constitutive *35S$_{pro}$* led to the highest phytoene level when compared with the seed-specific Gt-1$_{pro}$.

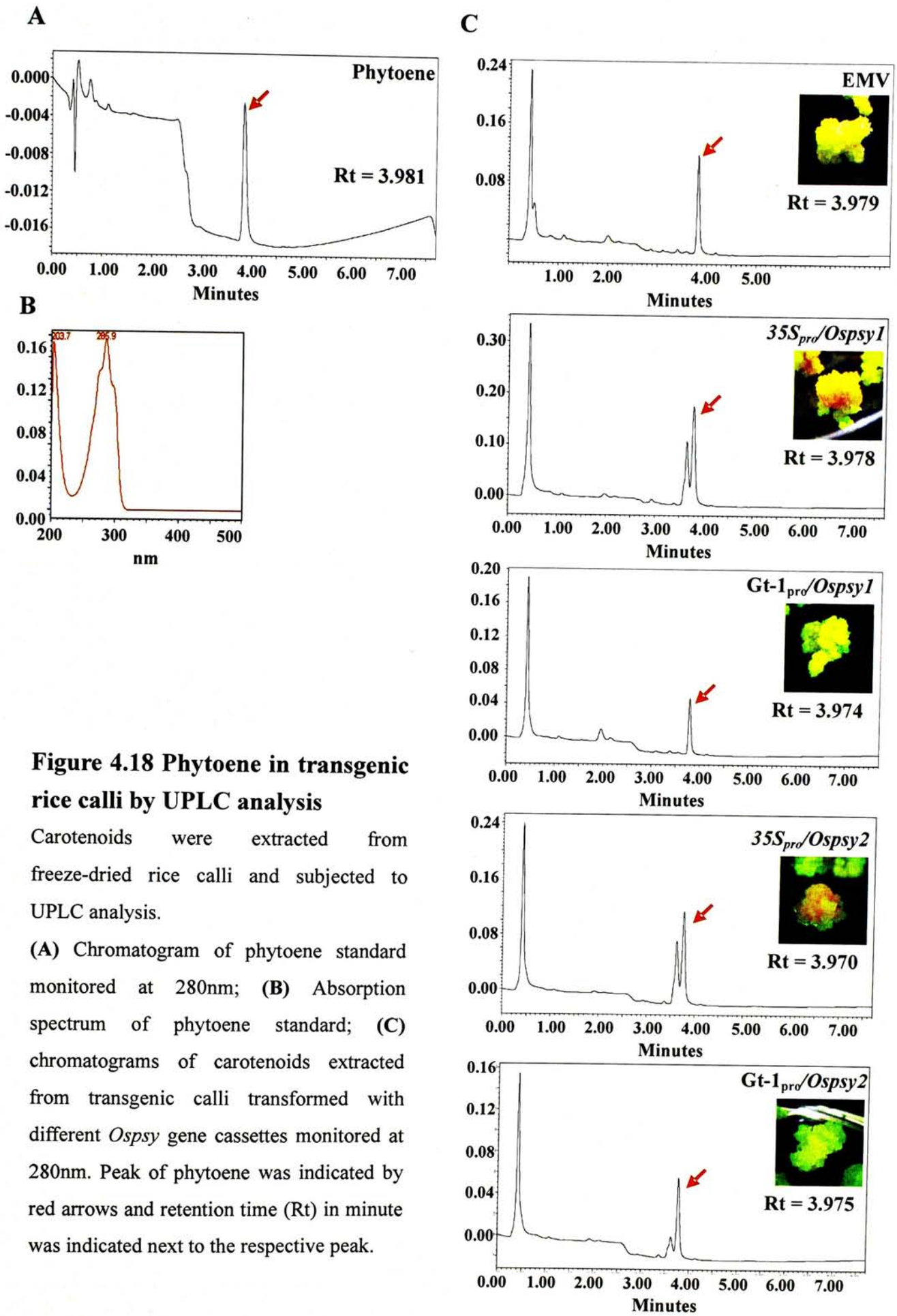


Figure 4.18 Phytoene in transgenic rice calli by UPLC analysis

Carotenoids were extracted from freeze-dried rice calli and subjected to UPLC analysis.

(A) Chromatogram of phytoene standard monitored at 280nm; **(B)** Absorption spectrum of phytoene standard; **(C)** chromatograms of carotenoids extracted from transgenic calli transformed with different *Ospsy* gene cassettes monitored at 280nm. Peak of phytoene was indicated by red arrows and retention time (Rt) in minute was indicated next to the respective peak.

As to β-carotene, it was only detected in the orange *35S$_{pro}$/Ospsy1* and *35S$_{pro}$/Ospsy2* calli, but not in the yellow EMV, Gt-1$_{pro}$/*Ospsy1* and Gt-1$_{pro}$/*Ospsy2* samples, as indicated by the peak with an arrow in the chromatograms in Figure 4.19. The β-carotene level of the orange *35S$_{pro}$/Ospsy1* and *35S$_{pro}$/Ospsy2* calli were 1475ng/mg and 793ng/mg of dry mass of calli respectively (p<0.05). This suggests that the constitutive expression of OsPSY1 resulted in a higher β-carotene accumulation in rice calli than OsPSY2. Also, the accumulation of β-carotene in the *35S$_{pro}$/Ospsy1* and *35S$_{pro}$/Ospsy2* calli could explain the orange appearance of these transgenic calli, indicating that both OsPSY1 and OsPSY2 were functional in rice calli.

The amounts of phytoene and β-carotene in each calli sample, and the average amounts for each construct were summarized in Table 4.2.

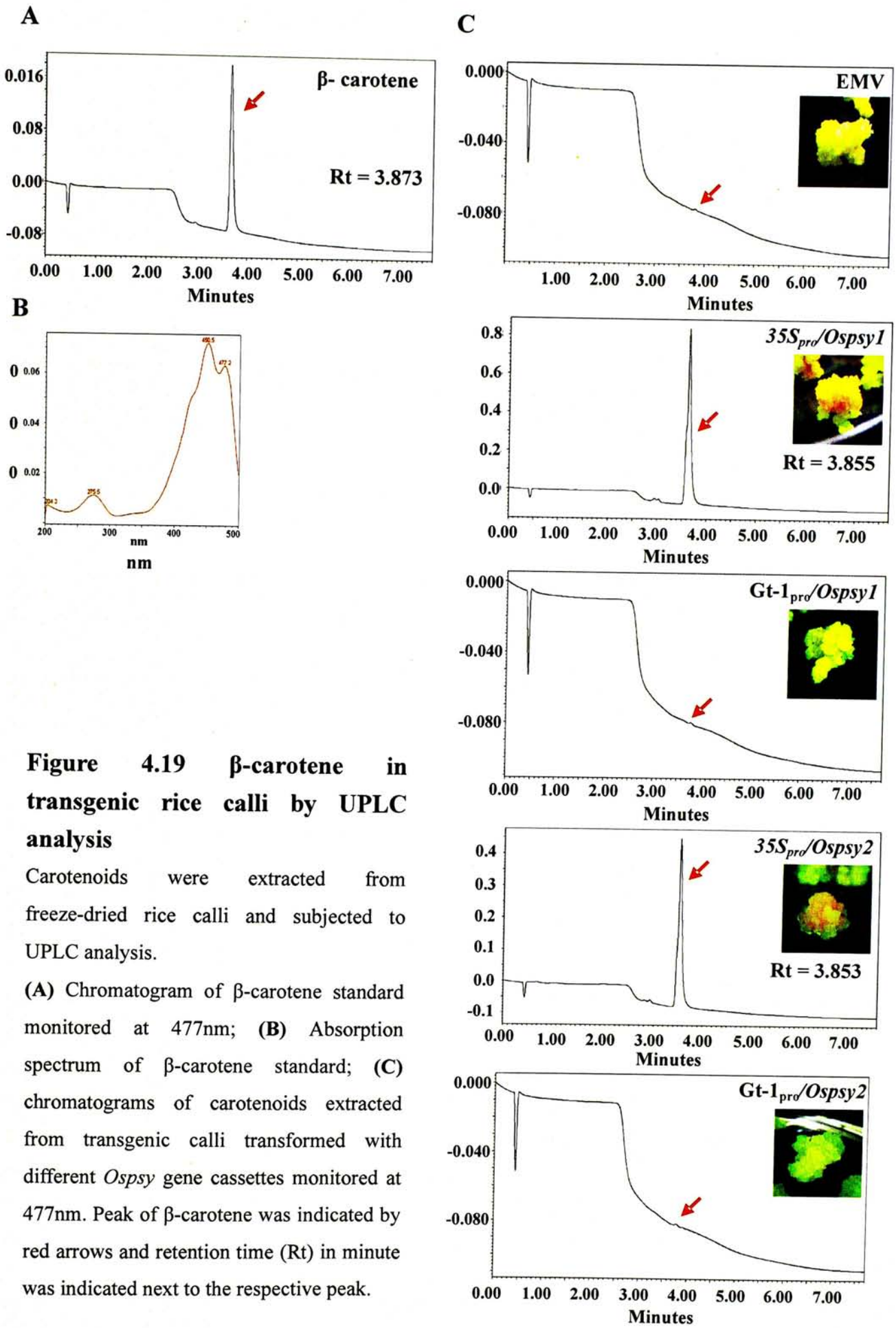


Figure 4.19 β-carotene in transgenic rice calli by UPLC analysis

Carotenoids were extracted from freeze-dried rice calli and subjected to UPLC analysis.

(A) Chromatogram of β-carotene standard monitored at 477nm; **(B)** Absorption spectrum of β-carotene standard; **(C)** chromatograms of carotenoids extracted from transgenic calli transformed with different *Ospsy* gene cassettes monitored at 477nm. Peak of β-carotene was indicated by red arrows and retention time (Rt) in minute was indicated next to the respective peak.

Table 4.2 Phytoene and β-carotene contents in transgenic calli harboring different gene constructs by UPLC analysis

Constructs	Plants	Phytoene content per dry mass (ng/mg)	Average phytoene content per dry mass (ng/mg)	β-carotene content per dry mass (ng/mg)	Average β-carotene content per dry mass (ng/mg)
EMV	E-01	447.10			
	E-02	753.80			
	E-03	336.79			
	E-04	887.01			
	E-05	650.66	**589.10 (SD = 173.52)**	**cannot be detected**	
	E-06	641.93			
	E-07	522.80			
	E-08	359.27			
	E-09	622.83			
	E-10	668.77			
35S$_{pro}$/Ospsy1	C1-01	3631.57		1888.66	
	C1-02	2665.36		1179.23	
	C1-03	4810.45		1399.59	
	C1-04	2588.63		1072.97	
	C1-05	3131.01	**5042.28 (SD = 2705.49)**	1559.02	**1474.53 (SD = 706.25)**
	C1-06	10479.01	**P value: 0.00055**	1442.08	
	C1-07	5049.92		1421.50	
	C1-08	3579.27		489.99	
	C1-09	5357.15		1101.85	
	C1-10	9130.40		3190.41	
Gt-1$_{pro}$/Ospsy1	G1-01	201.74			
	G1-02	421.56			
	G1-03	344.48			
	G1-04	321.34			
	G1-05	514.33	**387.95 (SD =145.17)**	**cannot be detected**	
	G1-06	501.55	**P value: 0.1179**		
	G1-07	513.70			
	G1-08	565.90			
	G1-09	373.29			
	G1-10	121.56			
35S$_{pro}$/Ospsy2	C2-01	1669.29		583.79	
	C2-02	4251.39		1225.40	
	C2-03	3604.44		1483.51	
	C2-04	3511.55		1795.99	
	C2-05	2480.08	**3499.78 (SD = 1385.99)**	480.37	**792.71 (SD = 596.15)**
	C2-06	5944.85	**P value: 0.00009**	1216.69	
	C2-07	5461.99		464.99	
	C2-08	2115.96		135.05	
	C2-09	3114.05		517.15	
	C2-10	2844.21		24.13	
Gt-1$_{pro}$/Ospsy2	G2-01	1503.69			
	G2-02	1718.79			
	G2-03	2794.85			
	G2-04	1560.17			
	G2-05	2510.71	**2417.53 (SD = 839.41)**	**cannot be detected**	
	G2-06	2421.15	**P value: 0.00006**		
	G2-07	1937.55			
	G2-08	2432.82			
	G2-09	2986.55			
	G2-10	4309.00			

Remarks: the phytoene and β-carotene contents of transgenic calli were calculated by comparing the corresponding peak areas with that of phytoene and β-carotene standard. The average carotenoid content was calculated. Average values lower than that of empty vector were marked in red while those with higher values in blue.

4.1.5.1.2 Carotenoid profiling of transgenic rice leaves

Carotenoid profiles of 54 transgenic rice leaf samples were determined. In order to eliminate the effect from different transgene copy numbers, leaf samples were collected only from transgenic plants with single copy of transgene. Carotenoids in the freeze-dried rice leaves were extracted and subjected to alkaline treatment to remove chlorophyll before UPLC analysis. β-carotene, violaxanthin and xanthophylls were followed at 477nm at different retention times. Summary of the amounts of β-carotene, violaxanthin and xanthophylls of each sample and the average amounts for each gene cassette were presented in Table 4.3.

The β-carotene content of leaves with *Ospsy2* construct was increased up to 12514ng/mg for $35S_{pro}$, and 15145ng/mg for Gt-1_{pro}, of dry mass of leaves; in contrast, those harboring *Ospsy1* constructs contained decreased β-carotene content amounting to 1190ng/mg for $35S_{pro}$, and 2545ng/mg for Gt-1_{pro}, of dry mass of leaves when compared to the EMV. All of these differences were statistically significant. This suggests that OsPSY2 could function properly in rice leaves, leading to an increase in β-carotene level while overexpression of *Ospsy1* led to a decrease.

Since $35S_{pro}$ could direct a stronger expression of *Ospsy1* in leaves than Gt-1_{pro}, the reduction in β-carotene level was more significant in the $35S_{pro}$/*Ospsy1* leaves. Chromatograms showing the detection of β-carotene content in leaves harboring different gene constructs were shown in Figure 4.20.

As for violaxanthin and xanthophylls, increased amounts of these carotenoids in leaf samples from Gt-1_{pro}/*Ospsy1*, *35S*$_{pro}$/*Ospsy2* and Gt-1_{pro}/*Ospsy2*, except *35S*$_{pro}$/*Ospsy1* were observed. However, all of these changes gave a *p*-value greater than 0.05, hence, they were not statistically important. As the actions of both *Ospsy1* and *Ospsy2* are separated by several enzymatic steps from the production of violaxanthin and xanthophylls in the carotenoid biosynthetic pathway, the effects of them have less direct impact on the violaxanthin and xanthophylls contents. Since the expression of *Ospsy1* driven by the seed-specific Gt-1_{pro} was significantly weaker than that of the *35S*$_{pro}$, the negative effect on carotenogenic activity by Gt-1_{pro}/*Ospsy1* could no longer influence the violaxanthin and xanthophylls amounts in leaves.

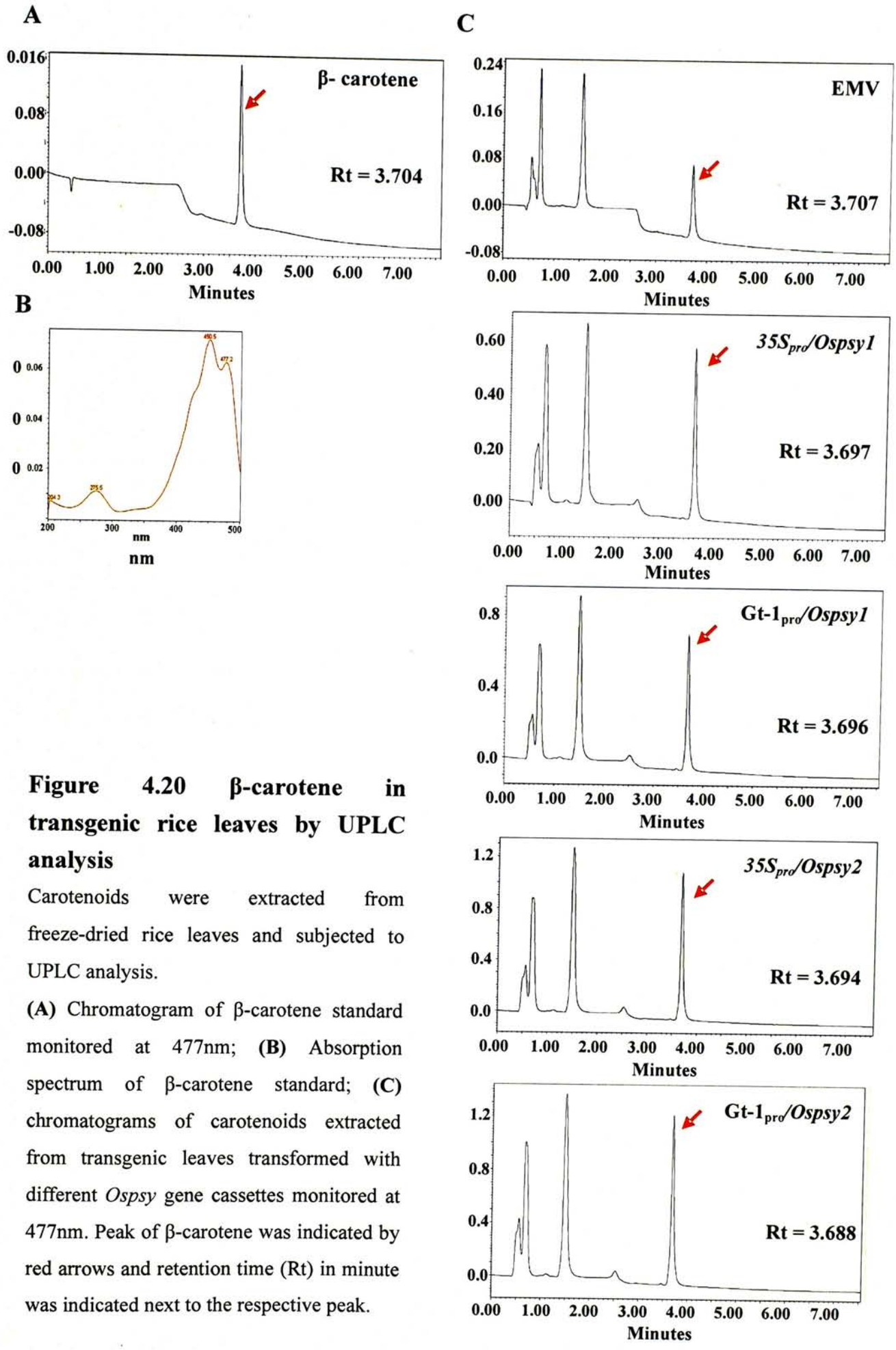


Figure 4.20 β-carotene in transgenic rice leaves by UPLC analysis

Carotenoids were extracted from freeze-dried rice leaves and subjected to UPLC analysis.

(A) Chromatogram of β-carotene standard monitored at 477nm; **(B)** Absorption spectrum of β-carotene standard; **(C)** chromatograms of carotenoids extracted from transgenic leaves transformed with different *Ospsy* gene cassettes monitored at 477nm. Peak of β-carotene was indicated by red arrows and retention time (Rt) in minute was indicated next to the respective peak.

Table 4.3 β-carotene, violaxanthin and xanthophylls content in transgenic leaves harboring different gene constructs by UPLC analysis

Constructs	Plants	Copy no.	β-carotene content per dry mass (ng/mg)	Average β-carotene content per dry mass (ng/mg)	Xanthophylls content per dry mass (ng/mg)	Average xanthophylls content per dry mass (ng/mg)	Violaxanthin content per dry mass (ng/mg)	Average violaxanthin content per dry mass (ng/mg)
EMV	E-01	0	812.25		4629.16		970.40	
	E-02	0	2410.38		9059.29		746.09	
	E-03	0	4454.24		4033.72		791.49	
	E-04	0	2338.89		8691.12		370.55	
	E-05	0	2230.60	**5625.71 (SD=3195.02)**	7086.47	**5275.01 (SD=1999.11)**	1260.91	**804.87 (SD=308.37)**
	E-06	0	7608.13		3797.12		470.02	
	E-07	0	7991.45		4160.32		1140.27	
	E-08	0	9218.65		4305.21		689.24	
	E-09	0	7608.13		3797.12			
	E-10	0	7991.45		4160.32			
	E-11	0	9218.65		4305.21			
$35S_{pro}$/*Ospsy1*	5-1	1	754.25		2681.75		367.29	
	9-2	1	808.24		3284.60		410.14	
	20-2	1	344.72		1391.12		189.30	
	105-1	1	2043.59	**1189.99 (SD=681.03)**	7259.76	**4536.13 (SD=2435.08)**	937.67	**545.41 (SD=280.66)**
	105-2	1	1268.30	**T-test: 0.000904**	5553.80	**T-test: 0.54**	572.85	**T-test: 0.13**
	107-1	1	1920.87		7045.75		795.18	
Gt-1_{pro}/*Ospsy1*	1-1	1	1504.72		5243.89		785.21	
	18-3	1	5360.87		20181.84		2699.11	
	30-1	1	1050.39		2321.37		761.55	
	54-2	1	2377.88	**2545.00 (SD=1453.83)**	8174.85	**9010.18(SD=5437.50)**	1826.91	**1278.73 (SD=717.87)**
	54-3	1	3605.46	**T-test: 0.011**	13559.21	**T-test: 0.052**	1876.33	**T-test: 0.07**
	54-5	1	2320.41		9798.95		1192.68	
	54-6	1	3333.79		9140.88		1021.05	
	54-7	1	983.00		3542.83		456.91	
	54-8	1	1883.74		7621.56		840.38	
	79-1	1	4379.74		15099.43		2018.78	
	79-2	1	1195.02		4427.20		587.15	
$35S_{pro}$/*Ospsy2*	8-1	1	9946.66		7048.40		1361.90	
	9-2	1	9223.84		4763.36		856.11	
	43-1	1	24824.92		12174.01		2345.73	
	81-2	1	6443.03	**12515.4 (SD=10573.93)**	2675.82	**6130.24 (SD=4772.83)**	637.63	**1100.82 (SD=887.76)**
	89-1	1	5586.97	**T-test: 0.02**	2900.53	**T-test: 0.54**	337.43	**T-test: 0.26**
	89-2	1	6247.04		3390.81		605.44	
	89-3	1	20634.91		9916.10		1868.43	
	89-4	1	2408.66		1266.72		222.34	
	93-2	1	43015.77		19466.60		3398.28	
	93-3	1	2605.41		1512.68		119.05	
	93-4	1	6925.24		3942.64		499.80	
	96-2	1	13235.55		5956.81		1265.80	
	96-3	1	17541.37		7796.39		1434.35	
	96-4	1	8849.30		4260.73		733.46	
	96-5	1	10243.22		4882.01		826.55	
Gt-1_{pro}/*Ospsy2*	66-2	1	10932.00		7070.35		1186.03	
	70-1	1	16768.18		7308.85		1230.90	
	70-2	1	26863.13		14801.04		1932.60	
	70-3	1	6289.37	**15145.29 (SD=12014.29)**	2704.63	**5782.08 (SD=3619.48)**	587.05	**955.51 (SD=467.70)**
	79-1	1	7365.95	**T-test: 0.027**	3125.20	**T-test: 0.69**	671.95	**T-test: 0.41**
	79-2	1	14370.14		6228.52		1135.81	
	79-3	1	15663.20		6048.07		1134.39	
	78-1	1	6223.57		2624.52		486.85	
	78-2	1	3073.02		1484.03		200.46	
	86-1	1	13602.40		5769.35		844.31	
	86-2	1	45447.23		6438.33		1100.21	

Remarks: the β-carotene, violaxanthin and xanthophylls contents of transgenic leaves were calculated by comparing the corresponding peak areas with that of β-carotene, violaxanthin and xanthophylls standard. The average carotenoid content was calculated. Average values lower than that of empty vector were marked in red while those with higher values in blue.

4.1.5.1.3 Carotenoid profiling of mature transgenic rice seeds

In order to study the effects of overexpression of *Ospsy1* and *Ospsy2* in rice seeds, carotenoids in mature dried T_1 rice seeds were extracted and subjected to UPLC analysis. Seeds from T_0 plant with single copy of transgene were used so as to eliminate variations due to different transgene copy numbers. Only phytoene was followed at 280nm as no subsequent carotenogenic enzymes were available to form other carotenoid species in rice endosperm.

Phytoene was detected in all seed samples as indicated by the peaks with arrows shown in Figure 4.21, including the EMV which did not contain any transgene. The minute amount of phytoene presented in EMV seeds may be due to the OsPSY activity in the seed coat during rice seed maturation.

All of the transgenic seeds with the gene cassettes showed statistically significant increases in phytoene level, but to different extents, as presented in Table 4.4. The overexpression of *Ospsy1* resulted in the greatest phytoene enhancement, by 140.1% (33.7ng/mg of dry endosperm in average) under the control of $35S_{pro}$ and by 145.7% (34.4ng/mg of dry endosperm in average) when controlled by the endosperm-specific $Gt\text{-}1_{pro}$. Whereas, overexpression of *Ospsy2* led to a lower increase by 32.1% (18.5ng/mg of dry endosperm in average) under the control of $35S_{pro}$ and 42.1% (19.9ng/mg of dry endosperm in average) when controlled by the $Gt\text{-}1_{pro}$. Though both OsPSY1 and OsPSY2 were functional in rice endosperm, the activity of OsPSY1

appeared to be much higher.

Generally, the Gt-1_{pro} driven constructs led to a slightly greater phytoene enhancement in rice endosperm, which may be due to the stronger seed-specific activity of Gt-1_{pro} than the constitutive *35S*$_{pro}$.

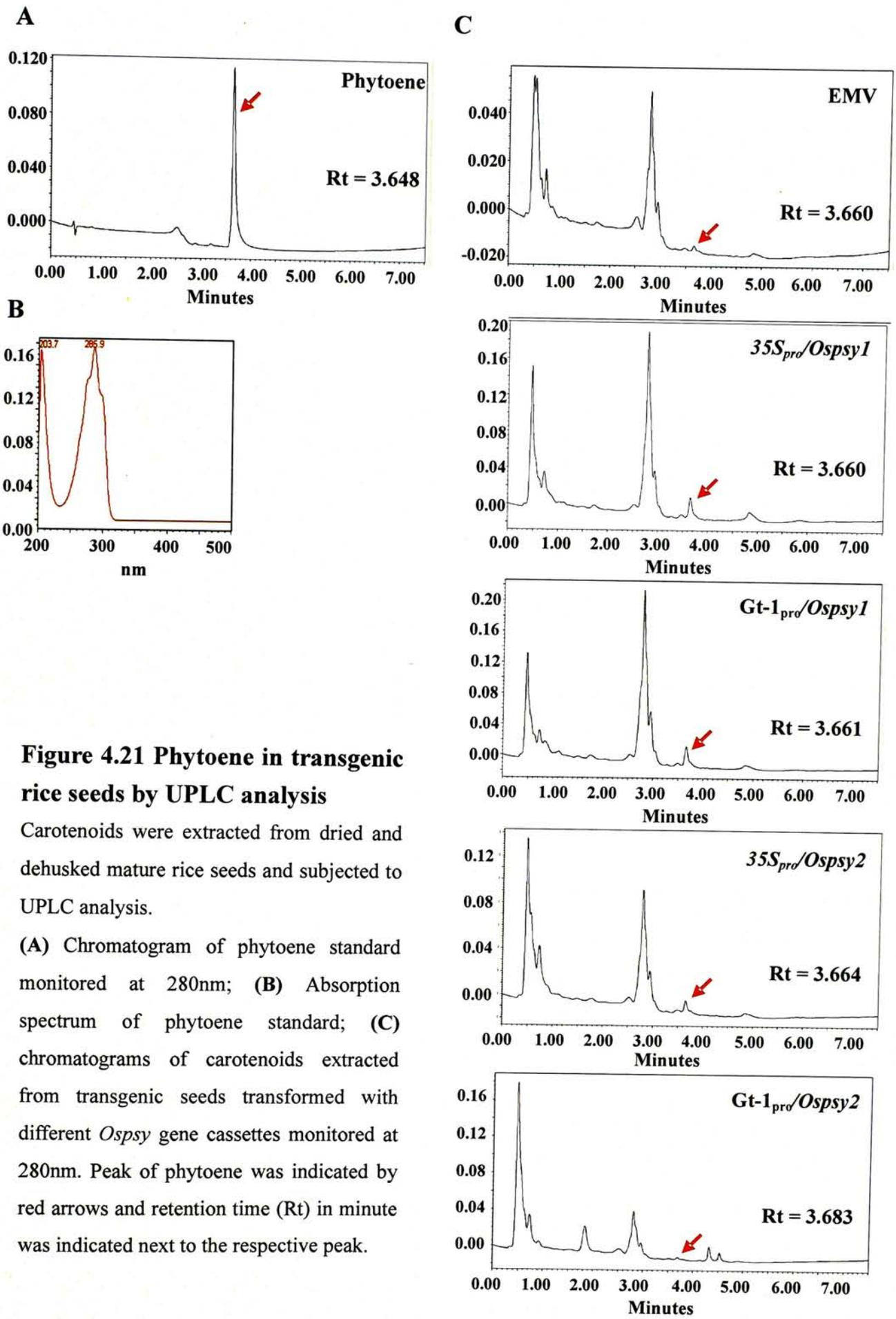


Figure 4.21 Phytoene in transgenic rice seeds by UPLC analysis

Carotenoids were extracted from dried and dehusked mature rice seeds and subjected to UPLC analysis.

(A) Chromatogram of phytoene standard monitored at 280nm; **(B)** Absorption spectrum of phytoene standard; **(C)** chromatograms of carotenoids extracted from transgenic seeds transformed with different *Ospsy* gene cassettes monitored at 280nm. Peak of phytoene was indicated by red arrows and retention time (Rt) in minute was indicated next to the respective peak.

Table 4.4 Phytoene content in transgenic seeds harboring different gene constructs by UPLC analysis

Constructs	Plants	Copy no.	Phytoene content per dry mass (ng/mg)	Average phytoene content per dry mass (ng/mg)
EMV	E-01	0	15.94	
	E-02	0	20.16	
	E-03	0	12.31	
	E-04	0	11.34	**14.0 (SD=2.9)**
	E-05	0	14.14	
	E-06	0	13.28	
	E-07	0	13.32	
	E-08	0	11.50	
$35S_{pro}$/*Ospsy1*	5-1	1	30.13	
	9-2	1	30.38	**33.74 (SD=4.75)**
	20-2	1	32.61	**T-test: 0.00017**
	105-1	1	33.79	
	105-2	1	41.78	
$Gt\text{-}1_{pro}$/*Ospsy1*	18-3	1	27.50	
	54-1	1	30.82	**34.39 (SD=6.58)**
	54-7	1	36.89	**T-test: 0.0057**
	95-2	1	42.35	
$35S_{pro}$/*Ospsy2*	8-1	1	15.09	
	9-2	1	15.74	
	43-1	1	18.69	
	89-1	1	15.99	
	89-2	1	23.89	**18.49 (SD=3.12)**
	89-4	1	18.13	**T-test: 0.0063**
	93-2	1	16.22	
	96-2	1	22.49	
	96-3	1	21.60	
	96-4	1	17.08	
$Gt\text{-}1_{pro}$/*Ospsy2*	70-2	1	16.21	
	70-3	1	20.85	
	70-4	1	20.06	**19.87 (SD=2.72)**
	79-1	1	23.69	**T-test: 0.0027**
	79-2	1	21.31	
	79-3	1	17.08	

Remarks: the phytoene contents of transgenic leaves were calculated by comparing the corresponding peak areas with that of phytoene standard. The average carotenoid content was calculated. Average values lower than that of empty vector were marked in red while those with higher values in blue.

4.2 Promoter analysis of modified rice *psy1* promoter

In order to elucidate the possibility to introduce the endosperm expression activity of the rice *psy1* promoter by introducing GCN4 multimer, the following five gene cassettes were constructed, so that the activity of the modified promoters can be determined with the GUS reporter gene, as depicted in Figure 4.22.

The effect of the GCN4 multimer can be determined, by comparing the GUS expression directed by $35S_{pro}m$/GUS and $35S_{pro}mG$/GUS. Besides, $Ospsy1(II)_{pro}G$/GUS was constructed to check the effect of GCN4 multimer on the TATA box of the *Ospsy1* promoter. Finally, the GUS expression directed by the $Ospsy1(I+II)_{pro}G$/GUS was determined to check whether the introduction of seven GCN4 motifs into the *Ospsy1* promoter could direct GUS expression in rice endosperm without affecting the leaf-specific activity of the native *Ospsy1* promoter.

Together with the empty vector harboring $35S_{pro}$/GUS six cassettes were transformed into *Japonica* rice 9983, and regenerated plants were subjected to the following analysis. First of all, genomic DNA PCR and Southern blot analysis were employed to screen for positive transformants and to check the copy number of transgene in the transgenic rice's genome. To determine the promoter activity in leaves and seeds of transgenic rice, histochemical staining and GUS activity assay were performed.

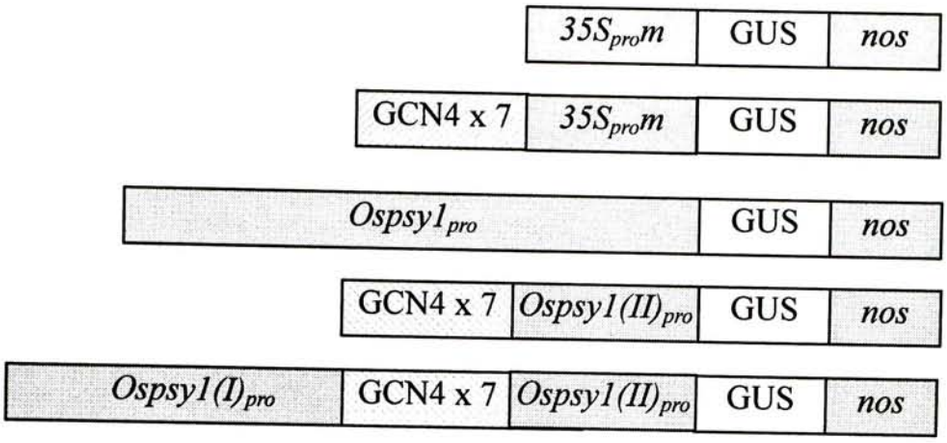


Figure 4.22 Schematic diagram of gene cassettes constructed for promoter analysis

Abbreviations:

35S$_{pro}$m	:	CaMV35S minimal promoter
Ospsy1$_{pro}$	:	1500bp rice phytoene synthase promoter
Ospsy1(I)$_{pro}$	:	1369bp rice phytoene synthase promoter
Ospsy1(II)$_{pro}$	:	131bp rice phytoene synthase promoter
GCN4 x 7	:	GCN4 multimer (or G in text)
GUS	:	GUS reporter gene

4.2.1 Construction of gene cassettes for promoter analysis

All of the four promoter fragments were cloned successfully. The 100bp *CaMV35S* minimal promoter was cloned from pBI221 using primer set B05 and B06, while the 1500bp $Ospsy1_{pro}$ was amplified using primer set B01 and B04 by PCR with wild type *Japonica* 9983 leaf genomic DNA. The partial $Ospsy1_{pro}$ fragments, 1369bp-Fragment I and 131bp-Fragment II, were cloned using primer sets B01 and B02, and B03 and B04 respectively. The GCN4 monomer was made by annealing the GCN4 oligos. After phosphorylation and ligation, GCN4 multimer was generated. The ligation product was resolved in 3% low melting agarose gel as shown in Figure 4.23 (C). Then, the fragments were cloned into pSB130/GUS vector forming the five constructs. After transformation into *E.coli* DH5α, the five constructs were isolated by mini-preparation and PCR amplification was performed to screen for positive transformant using B01 and S09 for $Ospsy1_{pro}$/GUS (~1600bp) and $Ospsy1(I+II)_{pro}G$/GUS (~1700bp); B03 and S09 for $Ospsy1(II)_{pro}G$/GUS (~400bp); B05 and S09 for $35S_{pro}m$/GUS (~300bp) and $35S_{pro}mG$/GUS (~400bp), as shown in Figure 4.23 (A). On the other hand, double digest was employed to screen for positive transformants. $Ospsy1(I+II)_{pro}G$/GUS and $Ospsy1_{pro}$/GUS were digested with EcoRI and NcoI, releasing a 1600bp and 1500bp fragment respectively; while $Ospsy1(II)_{pro}G$/GUS and $35S_{pro}m$/GUS were digested with HindII and NcoI, releasing fragments of 100bp, as presented in Figure 4.22 (B).

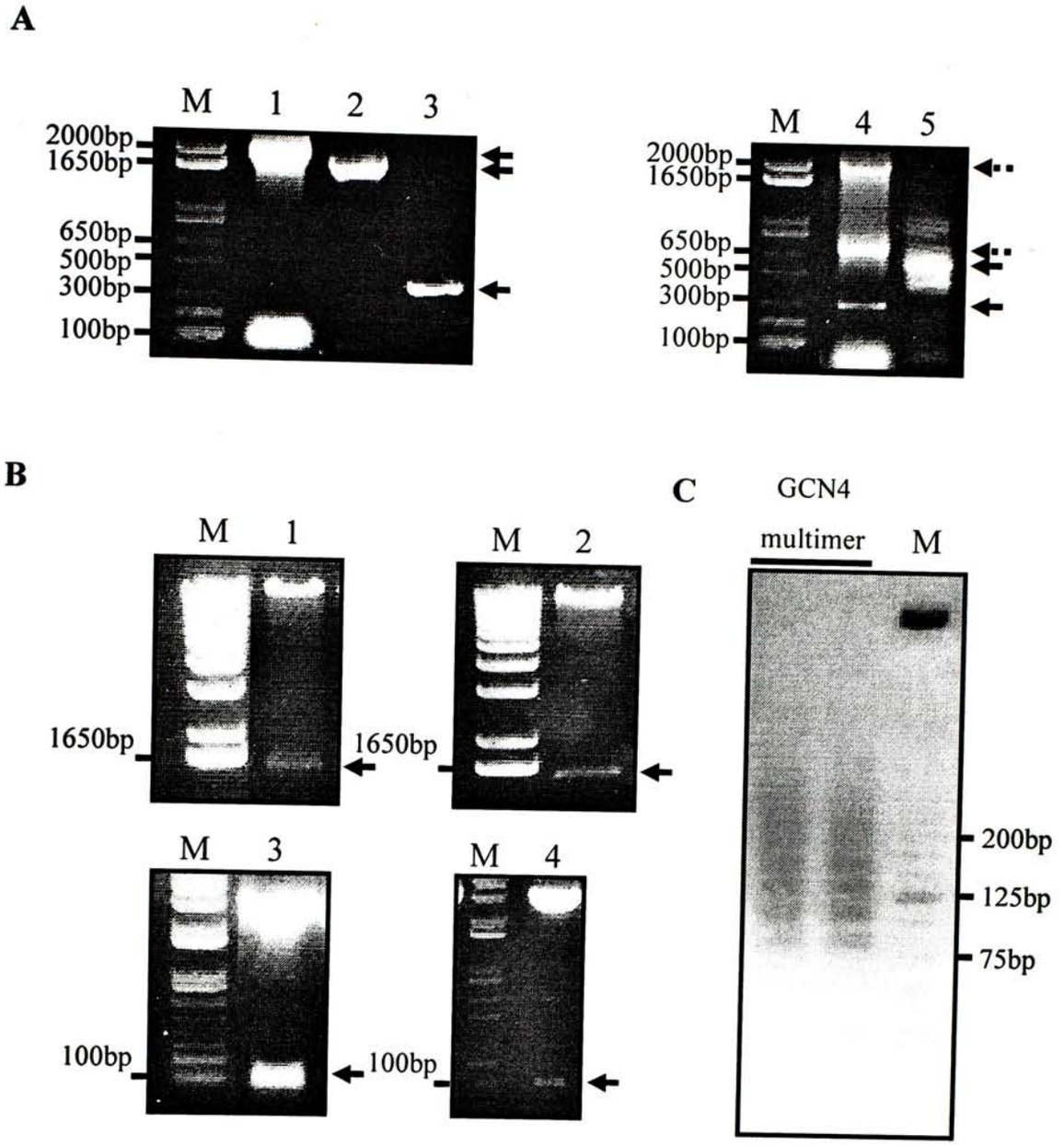


Figure 4.23 Confirmation of gene cassettes for promoter study

(A) Confirmation of gene cassettes in pSB130 vector by PCR. pSB130 vectors with gene cassettes were isolated and PCR were carried out for confirmation with different primer sets. Products were analyzed by gel electrophoresis. Lanes: M, 1kb plus DNA marker, Invitrogen; 1, *Ospsy1(I+II)*$_{pro}$*G*/GUS; 2, *Ospsy1*$_{pro}$/GUS; 3, *Ospsy1(II)*$_{pro}$*G*/GUS; 4, *35S*$_{pro}$*m*/GUS; and 5, *35S*$_{pro}$*mG*/GUS. **(B)** Confirmation of gene cassettes by double digestion. pSB130 vectors with gene cassettes were digested with different restriction enzymes and the digested products were analyzed by gel electrophoresis. Lanes: M, 1kb plus DNA marker, Invitrogen; 1, *Ospsy1(I+II)*$_{pro}$*G*/GUS with NcoI and EcoRI; 2, *Ospsy1*$_{pro}$/GUS with NcoI and EcoRI; 3, *Ospsy1(II)*$_{pro}$*G*/GUS with HindIII and NcoI; and 4, *35S*$_{pro}$*m*/GUS with HindIII and NcoI. **(C)** Formation of GCN4 multimer. GCN4 oligos were allowed to anneal. After phosphorylation, the product was resolved with 3% low melting agarose gel. Lanes: M, 25bp DNA ladder, Invitrogen; and GCN4 multimer product. Bands with expected size of promoter fragments were indicated with solid arrows while unidentified bands were indicated with dotted arrows.

4.2.2 Rice transformation

Primary calli were induced using mature rice seeds of *Japonica* 9983 and were co-cultivated with *Agrobacterium* harbouring the desired constructs. The treated calli were washed and transferred onto selection medium. During the selection stage, the calli were placed in darkness for 2 to 3 months. The non-transformed calli turned dark and died while the transformed cells formed resistant calli. A total of 590 groups of resistant calli formed were subjected to pre-regeneration and regeneration treatment. A total of 960 individuals transgenic rice were regenerated while 360 selected regenerated plants were planted in Gene Garden in April 2009.

4.2.3 Transgene detection

4.2.3.1 Genomic DNA PCR screening

Genomic DNA was extracted from the leaves of all regenerated rice before plantation. PCR was carried out to screen for regenerated plants harboring the transgene. Genomic DNA PCR was preformed using primer set B13 and B14 for $35S_{pro}$/GUS (empty pSB130/GUS vector), $Ospsy1_{pro}$/GUS, $Ospsy(I+II)_{pro}$/GUS, $Ospsy1(II)_{pro}G$/GUS constructs giving a 609bp product, and B15 and B16 for $35S_{pro}m$/GUS and $35S_{pro}mG$/GUS constructs giving a 1.3kb product. The PCR products were analyzed by gel electrophoresis as shown in Figure 4.24 to 4.27. A total of 360 plants with positive results were planted in Gene Garden.

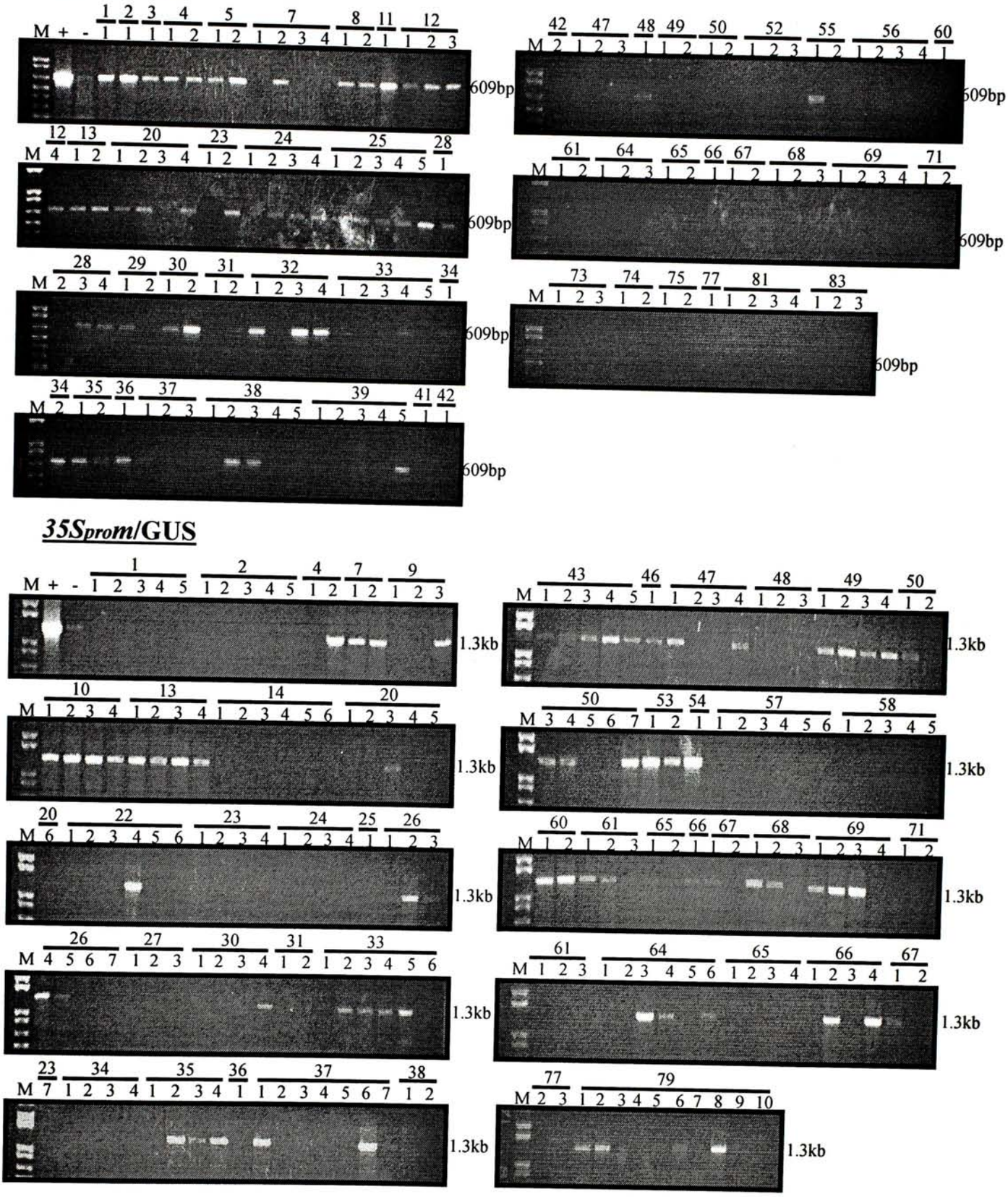


Figure 4.24 Genomic PCR of *35Spro*/GUS and *35Sprom*/GUS transgenic rice for promoter analysis

Genomic DNA was extracted from leaves of transgenic rice transformed with *35S$_{pro}$*/GUS and *35S$_{pro}$m*/GUS. PCR was performed using different primer sets. B13 and B14, and B15 and B16 were used for screening transgenic plants respectively. Products were analyzed by gel electrophoresis. Expected size of gene constructs *35S$_{pro}$*/GUS and *35S$_{pro}$m*/GUS respectively were 609 and 1.3kb. Lanes: M, 1kb plus DNA marker, Invitrogen; +, positive control using plasmid DNA of respective constructs; and -, negative control using untransformed plant.

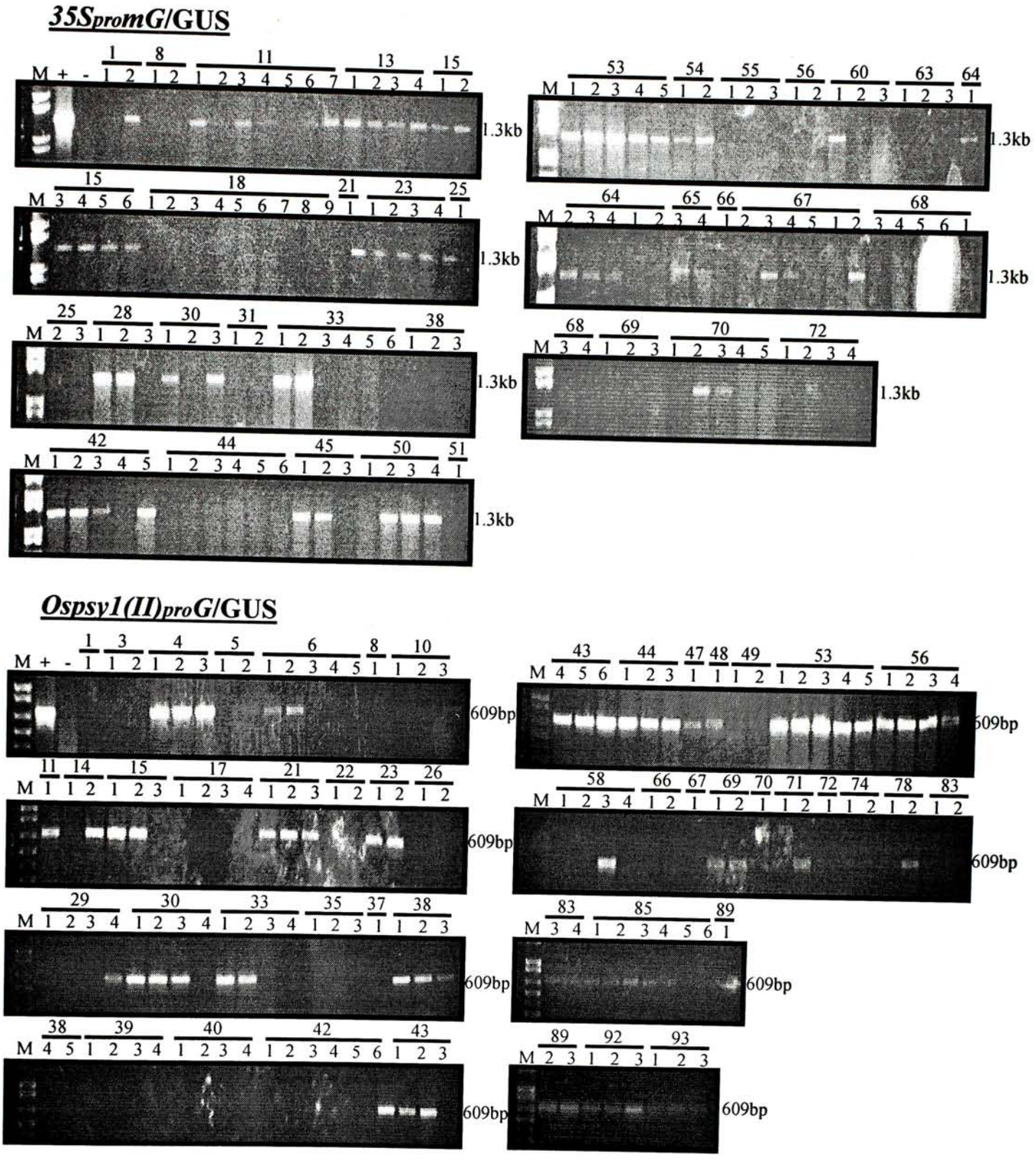


Figure 4.25 Genomic PCR of *35SpromG*/GUS and *Ospsy1(II)proG*/GUS transgenic rice for promoter analysis

Genomic DNA was extracted from leaves of transgenic rice transformed with $35S_{pro}mG$/GUS and $Ospsy1(II)_{pro}G$/GUS. PCR were performed using primer sets B15 and B16, and B13 and B14 for screening transgenic plants respectively. Products were analyzed by gel electrophoresis. Expected sizes of gene constructs were 1.3kb and 609bp for $35S_{pro}mG$/GUS and $Ospsy1(II)_{pro}G$/GUS respectively. Lanes: M, 1kb plus DNA marker, Invitrogen; +, positive control using plasmid DNA of respective constructs; and -, negative control using untransformed plant.

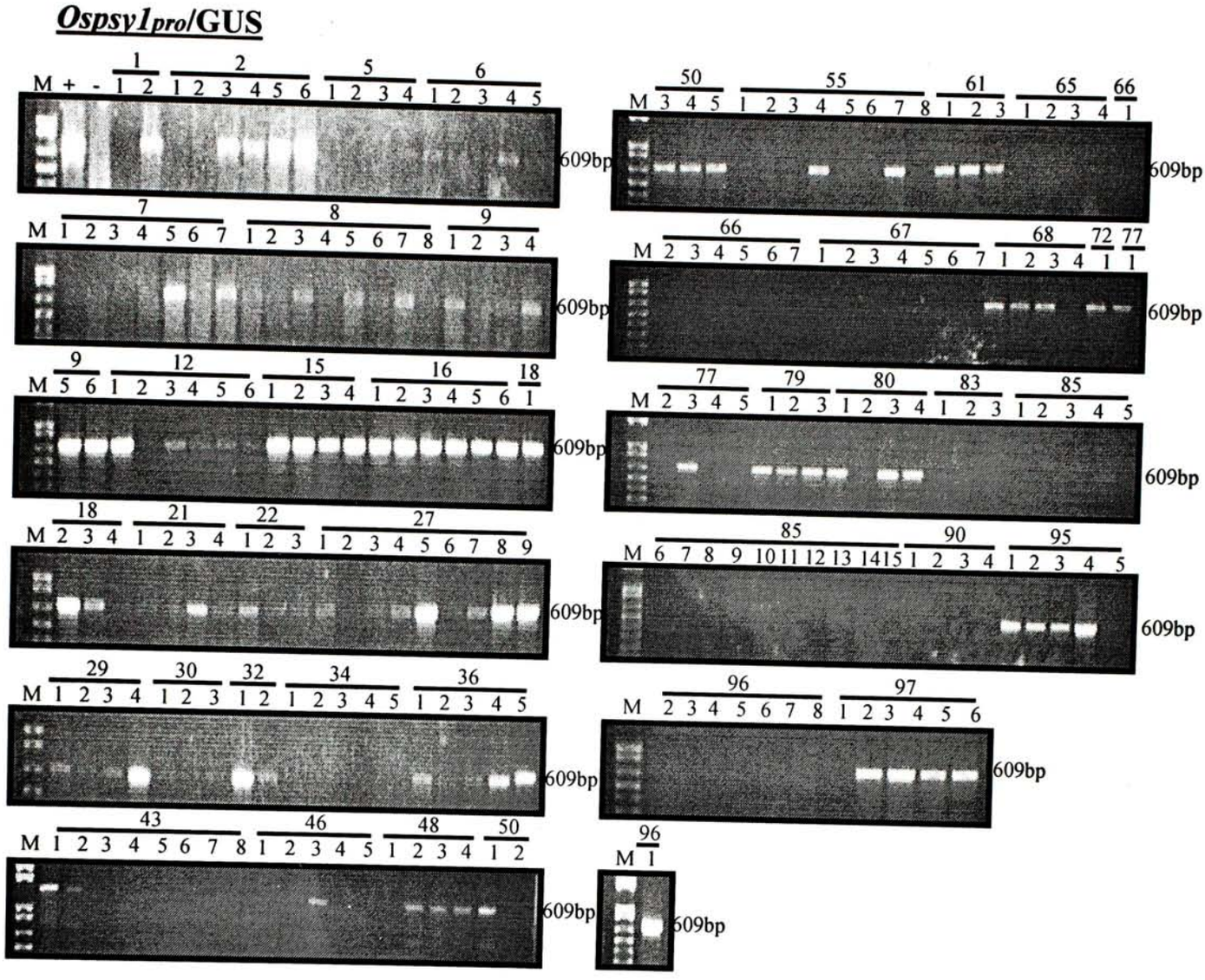


Figure 4.26 Genomic PCR of *Ospsy1pro*/GUS transgenic rice for promoter analysis

Genomic DNA was extracted from leaves of transgenic rice transformed with *Ospsy1*$_{pro}$/GUS. PCR was performed using primer sets B13 and B14 for screening transgenic plants. Products were analyzed by gel electrophoresis. Expected size of *Ospsy1*$_{pro}$/GUS was 609bp. Lanes: M, 1kb plus DNA marker, Invitrogen; +, positive control using plasmid DNA of respective constructs; and -, negative control using untransformed plant.

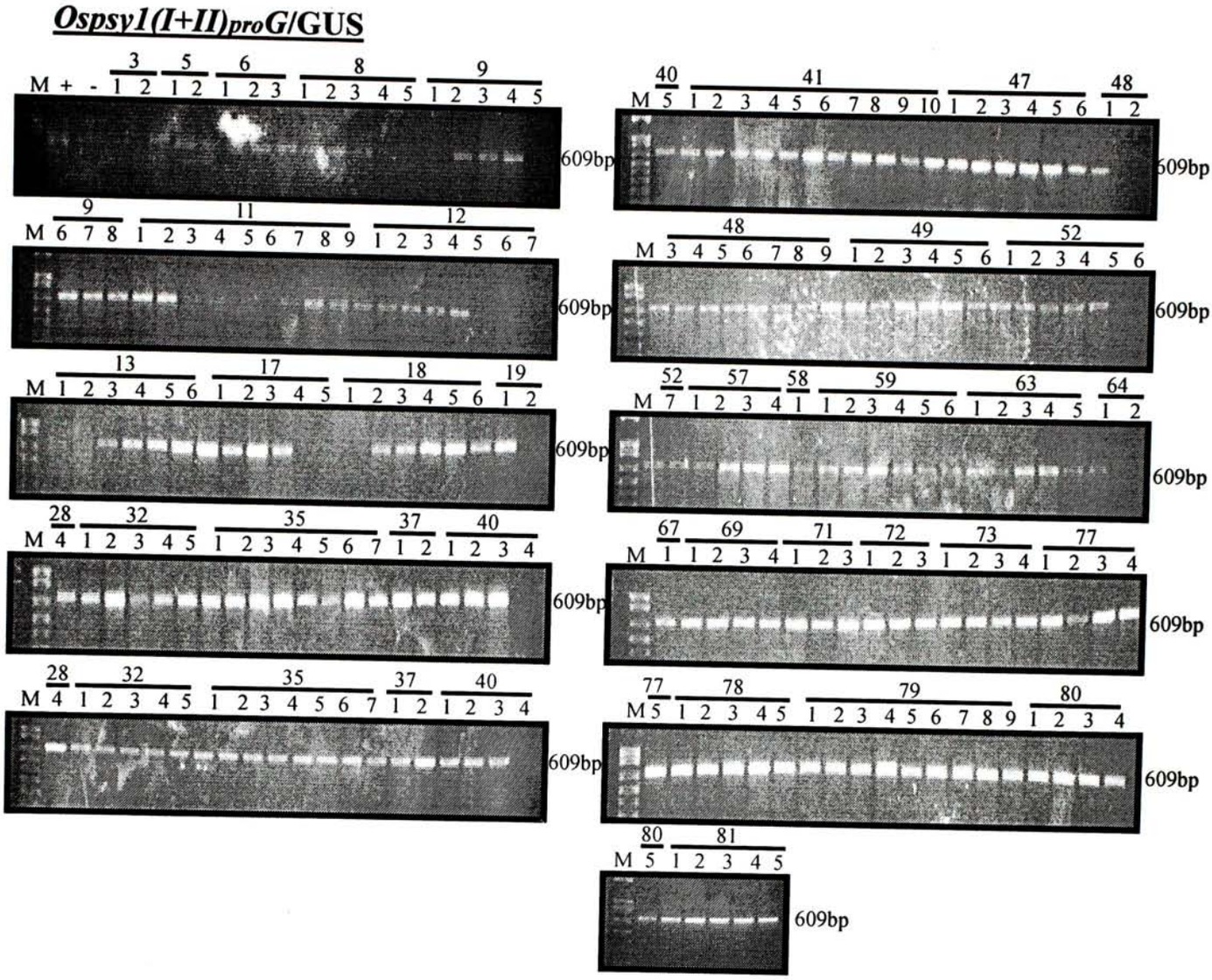


Figure 4.27 Genomic PCR of *Ospsy1(I+II)proG*/GUS transgenic rice for promoter analysis

Genomic DNA was extracted from leaves of transgenic rice transformed with *Ospsy1(I+II)$_{pro}$G*/GUS. PCR was performed using primer sets B13 and B14 for screening transgenic plants. Products were analyzed by gel electrophoresis. Expected size of *Ospsy1(I+II)$_{pro}$G*/GUS was 609bp. Lanes: M, 1kb plus DNA marker, Invitrogen; +, positive control using plasmid DNA of respective constructs; and -, negative control using untransformed plant.

4.2.3.2 Southern blot analysis

In order to determine the copy number of transgene in the genome of transgenic plants, Southern blot analysis was performed. DIG-labeled double-stranded DNA probes specific of GUS was made by PCR using primer sets P05 and P06. The probe made was subjected to dot blot analysis and the concentration was determined by comparing the intensity of the probe signals with known amounts of control DNA, as shown in Figure 4.28. The concentration of the GUS probe was 100ng/μl which was strong enough to be used in Southern blot analysis.

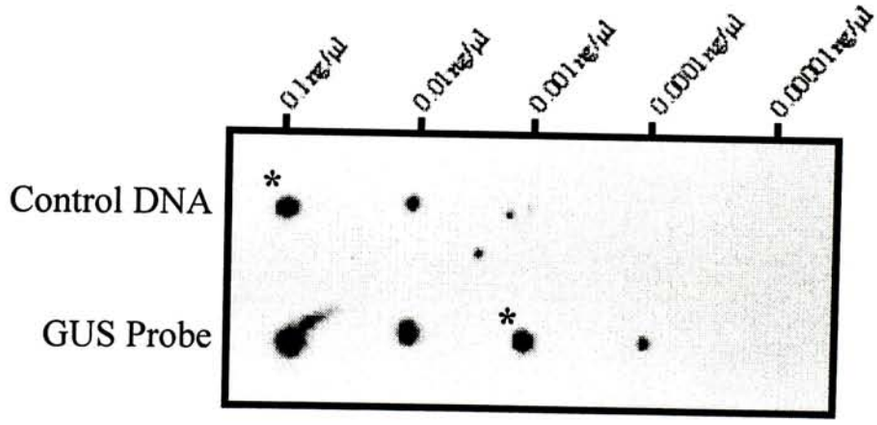


Figure 4.28 Dot blot analysis of DIG-labeled GUS DNA probes

DIG-labeled GUS DNA probes diluted to different concentrations were dotted on nylon membrane and detected with DIG Nucleic Acid Detection Kit (Roche Applied Science). Compared with known amounts of control DNA, the concentration of the GUS probe was 100ng/μl. Control DNA with intensity similar to that of DNA probes was marked with *.

4.2.3.2.1 Southern blot analysis of regenerated rice

A month after rice plantation, leaf samples were collected from all regenerated rice and genomic DNA was extracted. Southern blot analysis was performed to determine the copy number of transgene in the genome of every individual. Genomic DNA was extracted from leaves of $35S_{pro}$/GUS, $35S_{pro}m$/GUS, $35S_{pro}mG$/GUS, $Ospsy1_{pro}$/GUS, $Ospsy1(II)_{pro}G$/GUS and $Ospsy1(I+II)_{pro}G$/GUS plants. The DNA was subjected to NcoI digestion overnight and was resolved in 1% agarose gel. The DNA was transferred onto nylon membrane and detection was carried out using 500ng GUS probe. The Southern blot results for the leaves samples were shown in Figure 4.29 to 4.36.

Of the 211 regenerated plants with positive results, most harbored one to two copies of transgene, while only a few of them contained four to five copies. Details of transgenic rice regeneration and Southern blot analysis were summarized in Table 4.5.

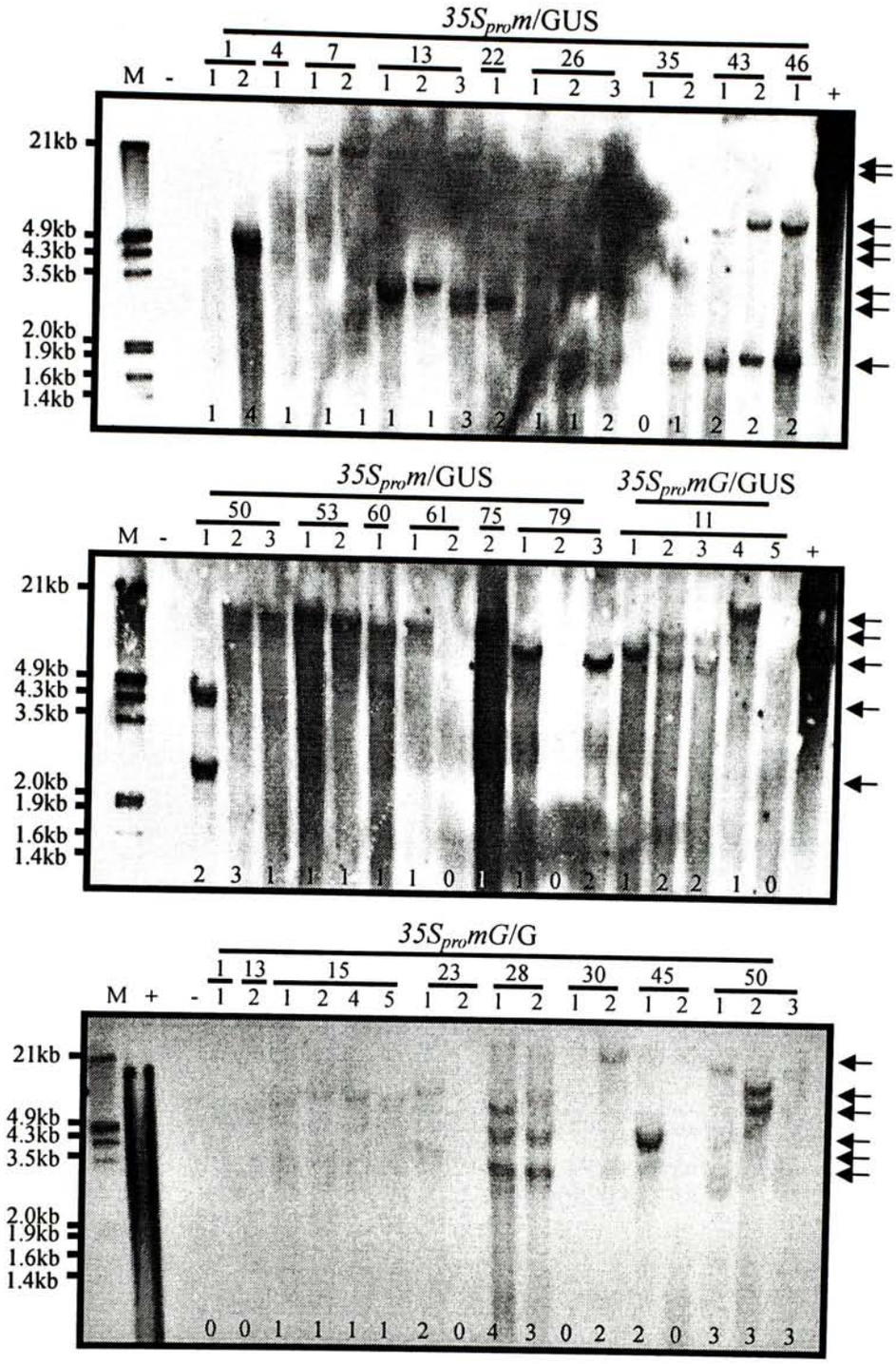


Figure 4.29 Southern blot analysis of *35S$_{pro}$m*/GUS and *35S$_{pro}$mG*/GUS transformed *Japonica* 9983 rice for promoter analysis

Genomic DNA extracted from transgenic rice leaves was digested with NcoI for *35S$_{pro}$m*/GUS, and *35S$_{pro}$mG*/GUS. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using GUS Probe. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; -, negative control; upper number refers to transgenic line and lower number refers to individual plants of each line.

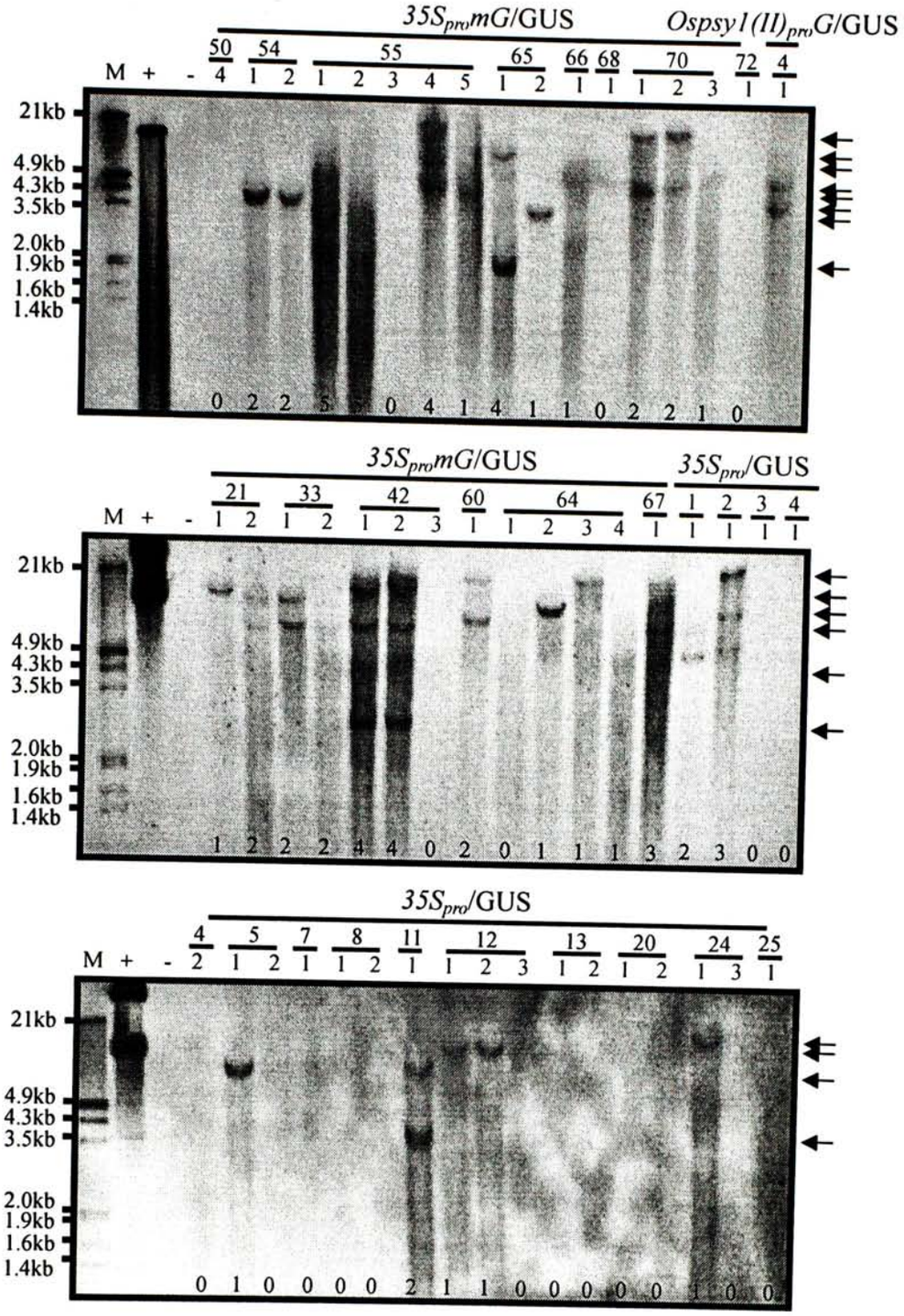


Figure 4.30 Southern blot analysis of *35S$_{pro}$*/GUS, *35S$_{pro}$mG*/GUS and *Ospsy1(II)$_{pro}$G*/GUS transformed *Japonica* 9983 rice for promoter analysis

Genomic DNA extracted from transgenic rice leaves was digested with NcoI for *35S$_{pro}$*/GUS, *35S$_{pro}$mG*/GUS, and *Ospsy1(II)$_{pro}$G*/GUS. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using GUS Probe. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; -, negative control; upper number refers to transgenic line and lower number refers to individual plants of each line.

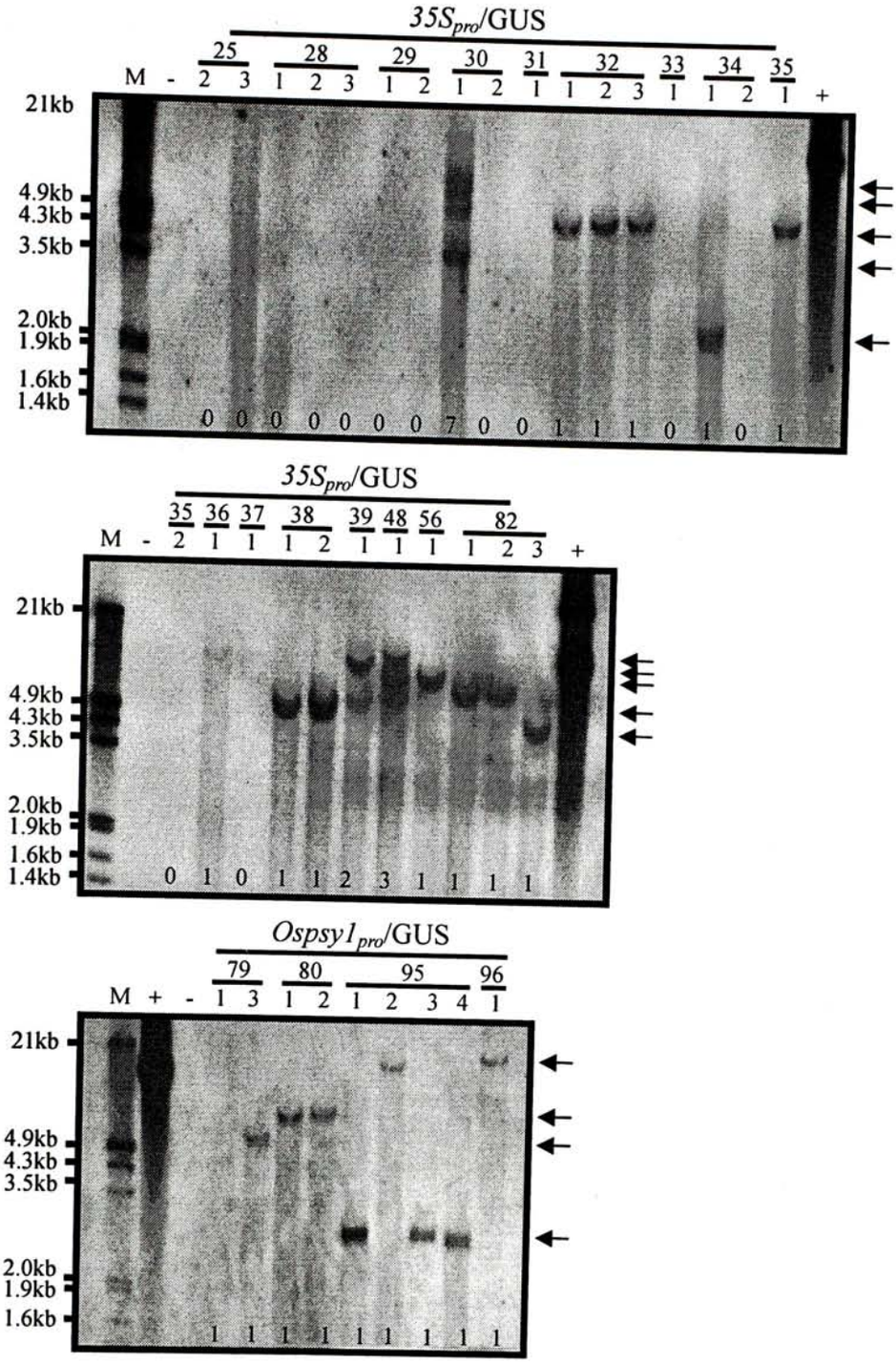


Figure 4.31 Southern blot analysis of *35S*$_{pro}$/GUS and *Ospsy1*$_{pro}$/GUS transformed *Japonica* 9983 rice for promoter analysis

Genomic DNA extracted from transgenic rice leaves was digested with NcoI for *35S*$_{pro}$/GUS, and *Ospsy1*$_{pro}$/GUS. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using GUS Probe. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; -, negative control; upper number refers to transgenic line and lower number refers to individual plants of each line.

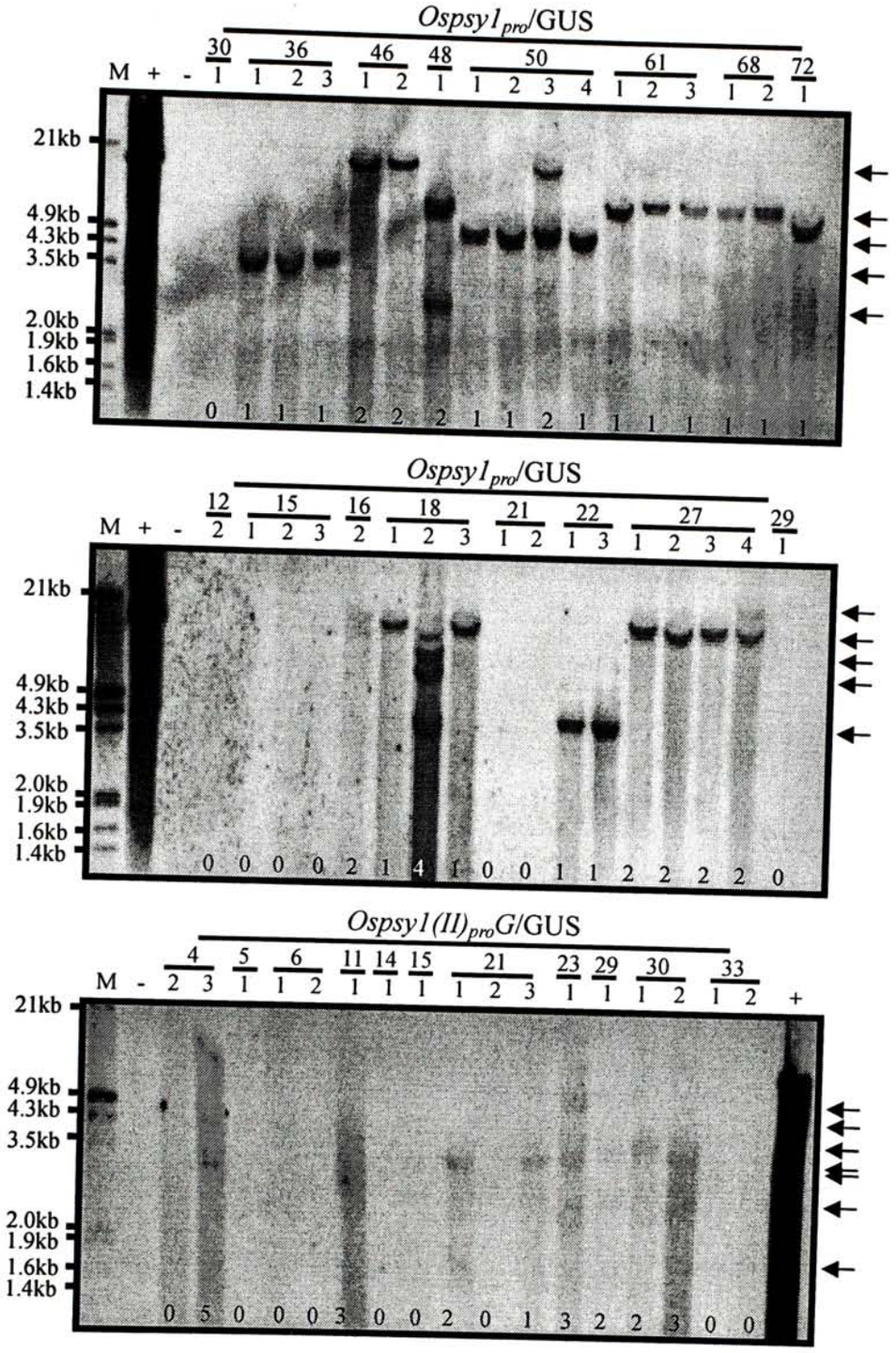


Figure 4.32 Southern blot analysis of *Ospsy1*$_{pro}$/GUS and *Ospsy1(II)*$_{pro}$*G*/GUS transformed *Japonica* 9983 rice for promoter analysis

Genomic DNA extracted from transgenic rice leaves was digested with NcoI for *Ospsy1*$_{pro}$/GUS, and *Ospsy1(II)*$_{pro}$*G*/GUS. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using GUS Probe. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; -, negative control; upper number refers to transgenic line and lower number refers to individual plants of each line.

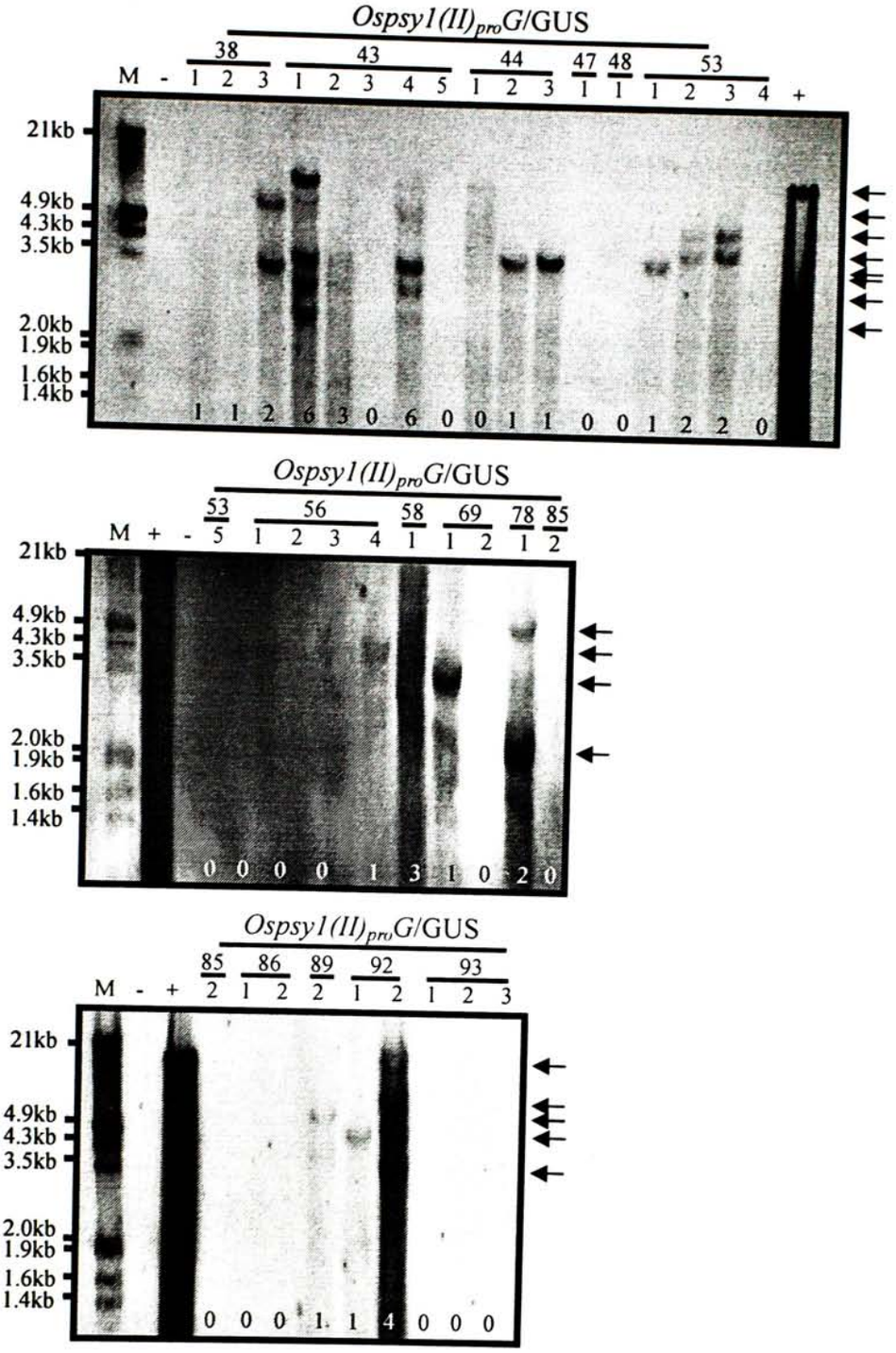


Figure 4.33 Southern blot analysis of *Ospsy1(II)$_{pro}$G*/GUS transformed *Japonica* 9983 rice for promoter analysis

Genomic DNA extracted from transgenic rice leaves was digested with NcoI for *Ospsy1(II)$_{pro}$G*/GUS. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using GUS Probe. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; -, negative control; upper number refers to transgenic line and lower number refers to individual plants of each line.

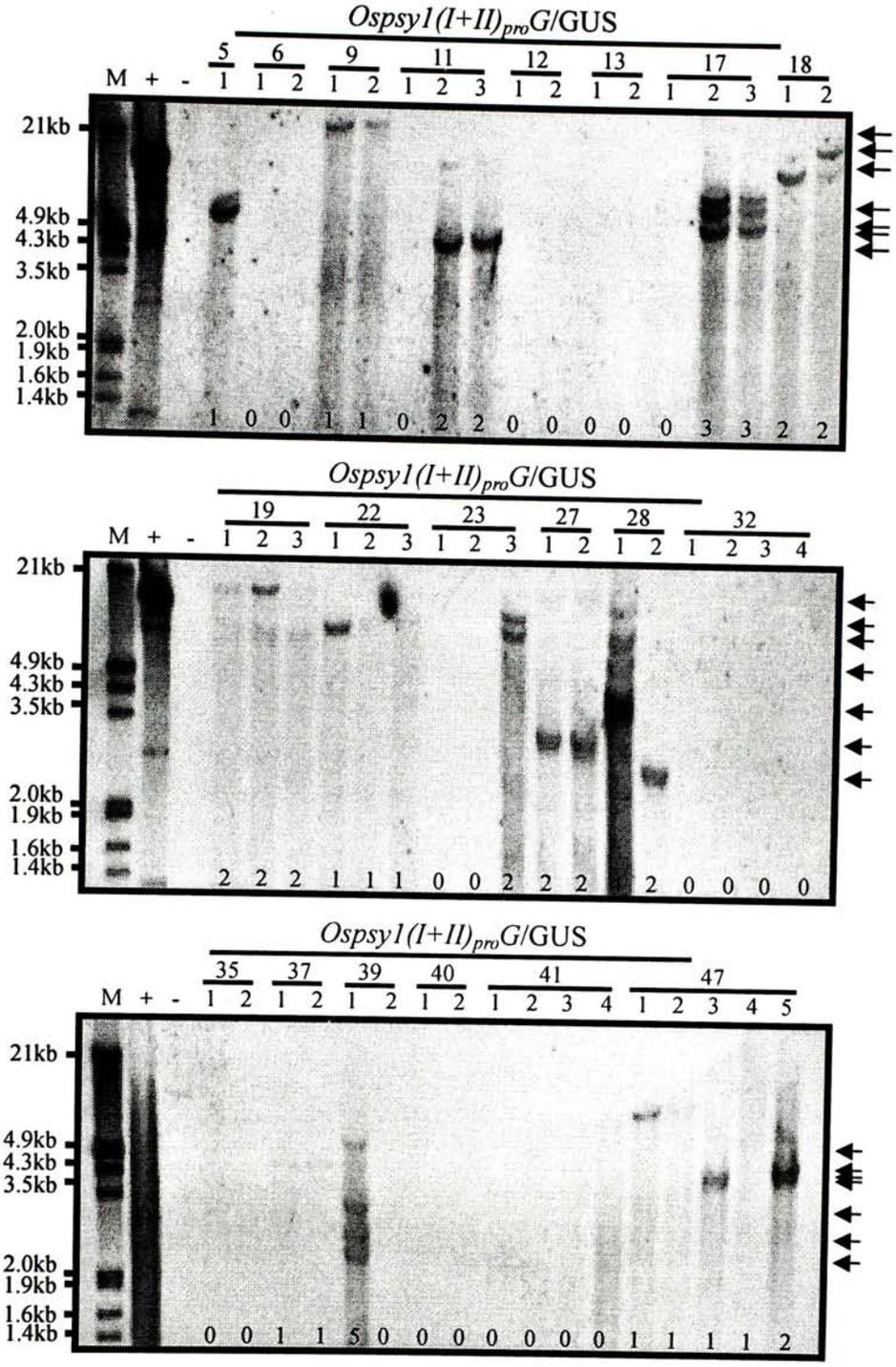


Figure 4.34 Southern blot analysis of *Ospsy1(I+II)$_{pro}$G*/GUS transformed *Japonica* 9983 rice for promoter analysis

Genomic DNA extracted from transgenic rice leaves was digested with NcoI for *Ospsy1(I+II)$_{pro}$G*/GUS. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using GUS Probe. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; -, negative control; upper number refers to transgenic line and lower number refers to individual plants of each line.

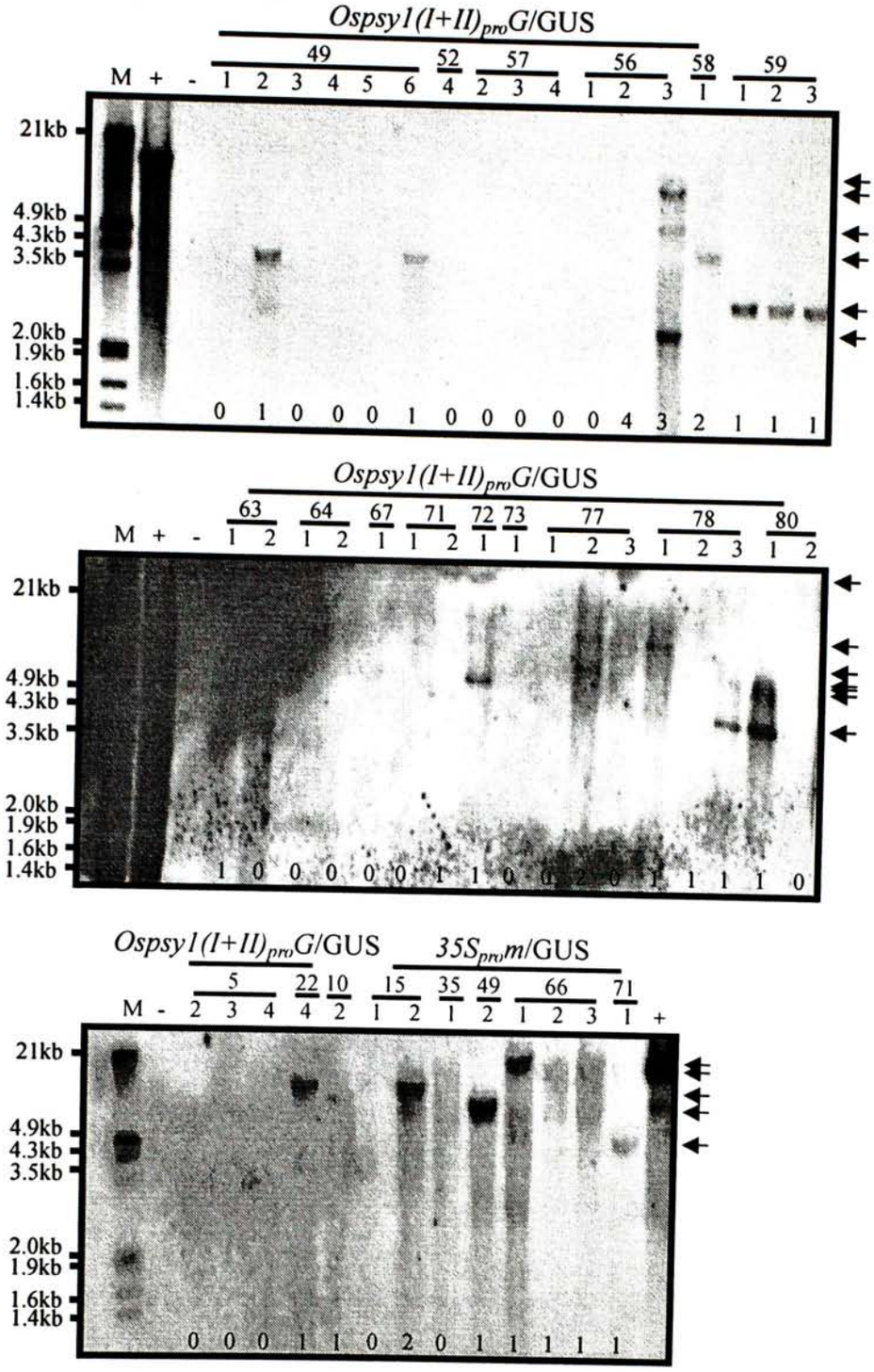


Figure 4.35 Southern blot analysis of *35S$_{pro}$m*/GUS and *Ospsy1(I+II)$_{pro}$G*/GUS transformed *Japonica* 9983 rice for promoter analysis

Genomic DNA extracted from transgenic rice leaves was digested with NcoI for *35S$_{pro}$m*/GUS, and *Ospsy1(I+II)$_{pro}$G*/GUS. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using GUS Probe. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; -, negative control; upper number refers to transgenic line and lower number refers to individual plants of each line.

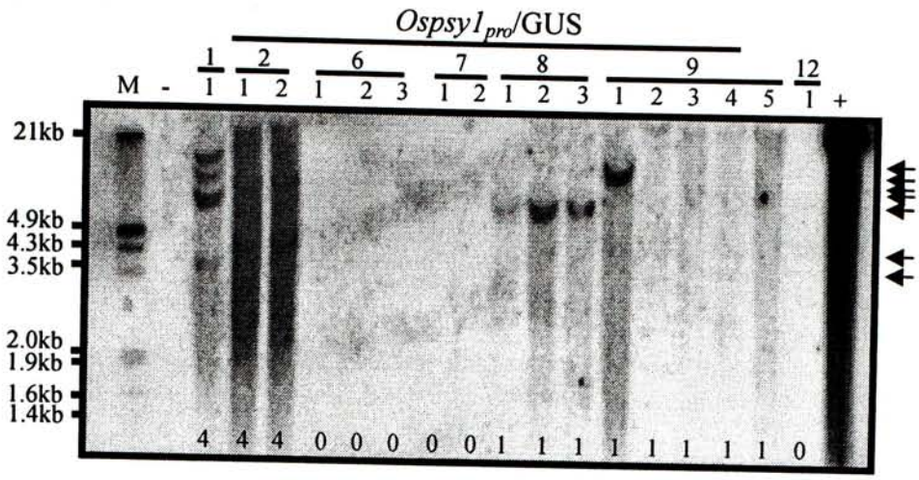


Figure 4.36 Southern blot analysis of *Ospsy1*$_{pro}$/GUS transformed *Japonica* 9983 rice for promoter analysis

Genomic DNA extracted from transgenic rice leaves was digested with NcoI for *Ospsy1*$_{pro}$/GUS. Digested products were resolved in 1% agarose gel and transferred onto nylon membrane. Detection was carried out using GUS Probe. Positive signals are indicated by arrows. Lanes: M, DNA Molecular Weight Marker III, DIG-labeled, Roche; +, positive control using respective plasmid DNA; -, negative control; upper number refers to transgenic line and lower number refers to individual plants of each line.

Table 4.5 Summary of regeneration of transgenic rice and copy number of transgene of different gene constructs

Constructs	Resistant calli	Regenerated tarnsgenic plants			Copy number of transgene				
		Number of line	Number of individual plants	Number of positive transformants planted (lines)	1	2	3	4	5
Empty vector	80	51	127	50 (29)	16	3	2	0	0
pSB130/*35S* $_{pro}$ *m* /GUS	85	64	181	45 (23)	22	8	3	0	0
pSB130/*35S* $_{pro}$ *m* G/GUS	85	33	126	55 (22)	15	13	5	5	2
pSB130/*Ospsy1* $_{pro}$ /GUS	110	40	203	72 (28)	32	9	0	4	0
pSB130/*Ospsy1 (II)* $_{pro}$ G/GUS	110	45	129	51 (24)	10	7	6	1	1
pSB130/*Ospsy1 (I+II)* $_{pro}$ G/GUS	120	40	194	87 (35)	28	14	2	2	1
Total number of plants grown				360					

4.2.4 Detection of promoter activity

4.2.4.1 Promoter activity in transgenic rice leaves

Ospsy1$_{pro}$ is a leaf-specific promoter which directs *Ospsy1* expression in rice leaves. Such activity may be influenced by the introduction of GCN4 multimer. In order to determine the effect of GCN4 on leaf-specificity and strength of the modified *Ospsy1*$_{pro}$, histochemical staining of GUS and GUS activity assay were performed on transgenic leaves. Leaves of 10 individual regenerated plants of each construct were collected. Each piece was divided into two parts: one half was used for histochemical staining while the other half was used in GUS activity assay.

4.2.4.1.1 Histochemical staining of GUS

The leaves samples were incubated with GUS staining solution overnight, and were washed with 70% ethanol until most of the chlorophyll was removed. The stained leaves samples were presented in Figure 4.37.

In wild type *Japonica* 9983 leaves no blue foci was developed indicating the absence of GUS in wild type rice leaves. Seven out of 10 *35S*$_{pro}$/GUS leaves were stained blue. Blue foci were developed throughout the leaves and the intensity was strong. As for the *35S*$_{pro}$*m*/GUS construct, the TATA box of *35S*$_{pro}$ only directed much weaker GUS expression in the leaves. In contrast, with the introduction of GCN4

multimer, for the *35S$_{pro}$mG*/GUS samples, 6 out of 10 leaves shown GUS expression at a stronger level than those of *35S$_{pro}$m*/GUS, suggesting that the addition of GCN4 to the TATA box enhances the GUS expression in leaves.

As for the leaf-specific *Ospsy1$_{pro}$*, 7 out of 10 leaves samples from *Ospsy1$_{pro}$*/GUS plants were stained blue while 5 of them showed strong GUS expression throughout the tissues. In contrast, the TATA box of *Ospsy1$_{pro}$* directed much weaker GUS expression in leaves while only 3 samples from *Ospsy1(II)$_{pro}$G*/GUS gave weak GUS signals. Finally, only 2 leaves samples from *Ospsy1(I+II)$_{pro}$G*/GUS were stained, with a signal much stronger than that of *Ospsy1(II)$_{pro}$G*/GUS.

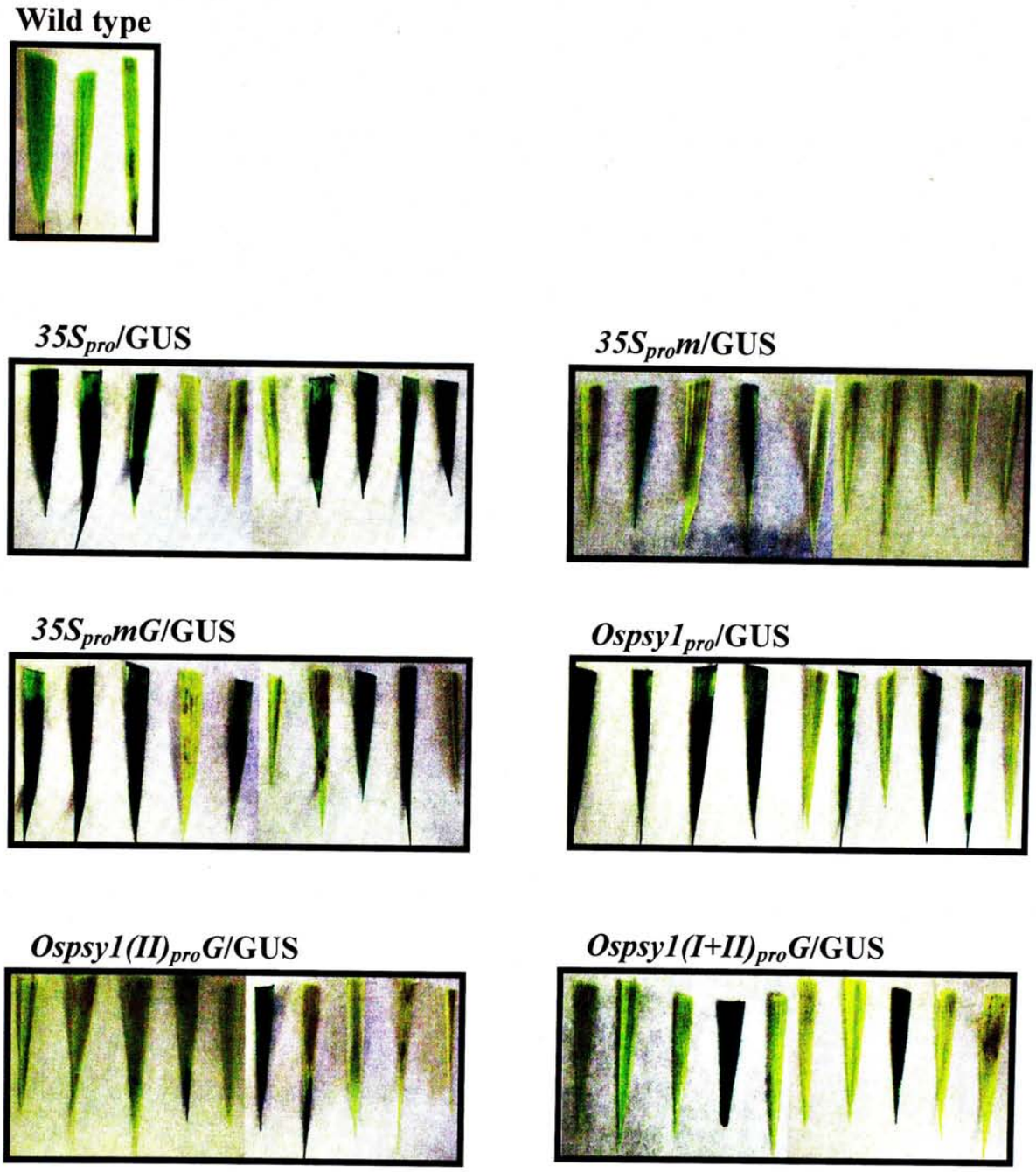


Figure 4.37 Histochemical staining of GUS activity in leaves from wild type and transgenic rice for promoter analysis

Ten pieces of leaves from wild type and transgenic *Japonica* 9983 rice were collected and were subjected to histochemical staining for 24 hours. Stained tissues were washed with 70% ethanol overnight. Blue foci developed indicated the expression of GUS.

4.2.4.1.2 GUS activity assay

GUS activity assay was performed using leaves samples collected from transgenic rice with positive transgene signals during Southern blot analysis. Total protein was extracted from the leaf samples and quantified by Bradford method. Ten microlitre of the plant extract was subjected to GUS activity assay. The GUS activity of each sample was calculated by comparing the fluorescence signal emitted by the samples to that of the 4-MUG standards. GUS activity of the leaf samples detected was presented in Figure 4.38, and the details were summarized in Table 4.6.

The assay revealed that the GUS activities driven by the $35S_{pro}m$, $35S_{pro}mG$ and $Ospsy1(II)_{pro}$ were close to the background level detected in wild type, with the *p*-values greater than 0.05 (*p*-value: 0.07, 0.109 and 0.055 respectively); thus, there was no statistically significant GUS expression by these three promoters. In contrast, the highest GUS activities were detected in leaves with $35S_{pro}$, followed by the $Ospsy1_{pro}$ and the modified $Ospsy1(I+II)_{pro}G$ with *p*-values of 0.03, 0.03 and 0.008, which were lower than 0.05. Thus there was statistically significant GUS expression driven by these three promoters. The GUS activity directed by $Ospsy1(I+II)_{pro}G$ was compared with that of $Ospsy1_{pro}$, and the *p*-value was greater than 0.05 (*p*-value: 0.19), indicating that there was no statistically significant difference between the activity of the two promoters. This suggests that the introduction of GCN4 multimer did not impose great impact on the strength and leaf-specificity of the native $Ospsy1_{pro}$.

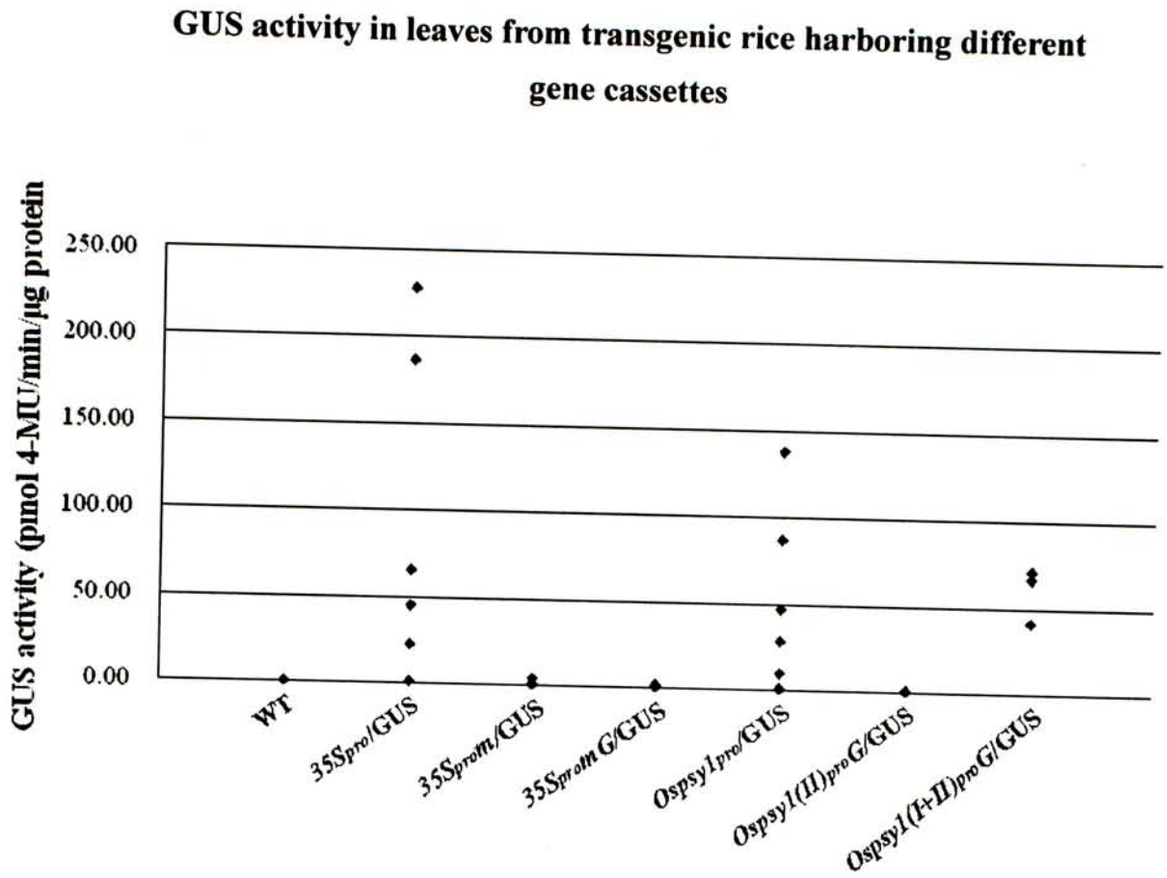


Figure 4.38 GUS activity of total protein from leaves of individual plants harboring different constructs

Each point represents the average GUS activity detected of a single leaf sample with three technical repeats. *p*-values of GUS activity compared with wild type (WT) were 0.03, 0.07, 0.109, 0.055, 0.03 and 0.008 respectively.

Table 4.6 Summary of GUS activity detected in leaves harboring different gene constructs

Remarks: GUS activity calculated with negative value was marked in red in blanket.

Constructs	Plants	Copy number of transgene	GUS activity (pmol 4-MU/min/μg protein)	GUS activity per copy number (pmol 4-MU/min/μg protein)	Standard deviation
Wild type	W-01		0.09		
	W-02		0.07		
	W-03		0.00		0.05
$35S_{pro}$/GUS	5-1	1	227.13	227.13	
	11-1	2	1.07	0.54	
	38-1	1	22.65	22.65	
	38-2	1	44.68	44.68	
	56-1	1	65.72	65.72	
	82-1	1	186.03	186.03	92.95
$35S_{pro}m1$/GUS	7-1	1	0.45	0.45	
	13-1	1	0.55	0.55	
	10-2	1	0.50	0.50	
	53-1	1	0.52	0.52	
	46-1	1	0.53	0.53	
	60-1	1	0.56	0.56	
	66-1	1	0.53	0.53	
	70-1	5	0.60	0.12	
	71-1	1	3.86	3.86	1.14
$35S_{pro}mG1$/GUS	15-1	1	1.72	1.72	
	11-4	1	1.62	1.62	
	55-5	1	0.71	0.71	
	50-1	3	0.76	0.25	
	60-2	2	0.72	0.36	
	28-2	3	0.61	0.20	0.69
$Ospyl_{pro}$/GUS	8-1	1	0.65	0.65	
	8-2	1	46.45	46.45	
	18-2	4	111.57	27.89	
	22-1	1	9.59	9.59	
	16-2	1	137.86	137.86	
	27-1	2	0.64	0.32	
	95-3	1	0.65	0.65	
	72-1	1	87.14	87.14	
	46-2	2	1.59	0.80	48.55
$Ospyl(II)_{pro}G1$/GUS	4-1	3	0.53	0.18	
	43-4	6	0.59	0.10	
	21-3	1	0.63	0.63	
	38-1	1	0.60	0.60	
	53-2	2	0.67	0.34	
	93-1	6	0.55	0.09	0.24
$Ospyl(I+II)_{pro}G1$/GUS	5-1	1	41.13	41.13	
	23-3	2	132.75	66.38	
	78-1	1	71.57	71.57	16.28

4.2.4.2 Promoter activity in transgenic immature seeds

GCN4 is a seed-specific *cis*-acting regulatory element in rice promoter. In order to introduce the seed expression activity to $Ospsy1_{pro}$, seven GCN4 were inserted within 200bp upstream of the TATA box of the $Ospsy1_{pro}$. To determine the effect of GCN4 on the modified $Ospsy1_{pro}$, histochemical staining of GUS and GUS activity assay were performed on transgenic immature seeds.

4.2.4.2.1 Histochemical staining of GUS

Transgenic immature seeds at 20 DAF were collected and dissected transversally for histochemical staining by the same treatment employed for leaf samples. The stained tissues were observed under low magnification with light microscope and high magnification with fluorescence microscope after being embedded in paraffin block.

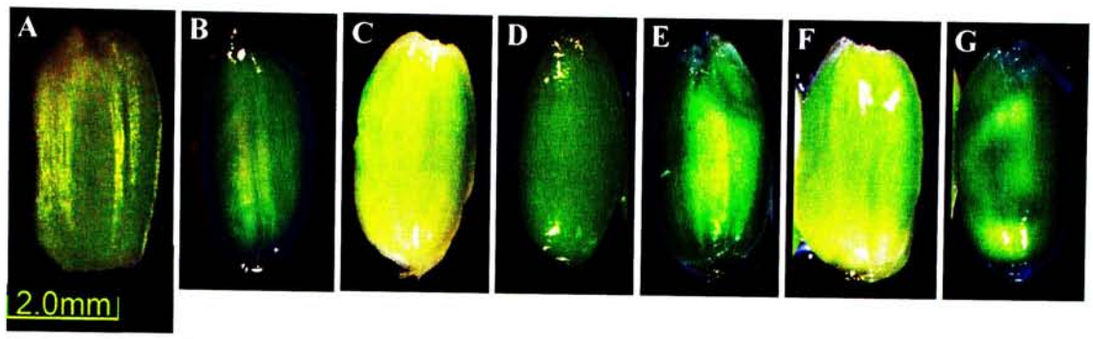
Under lower magnification (Figure 4.39), no tissue in wild type immature seed (A) could be stained, indicating the absence of GUS activity in wild type rice seeds. When considering the seed coats (upper panel), seed coat of immature seeds from the constitutive $35S_{pro}$ showed the greatest GUS expression (B) while seed coats of seeds from leaf-specific $Ospsy1_{pro}$ and modified $Ospsy1(I+II)_{pro}G$ were moderate in GUS expression (E) and (G). Seed coats of the seeds from the rest of constructs were not stained, revealing the absence of GUS expression in these tissues.

As for the embryo and endosperm (bottom panel), seeds of $35S_{pro}$ showed the

greatest GUS expression. GUS expression was strongest in the embryo and the central part of the endosperm (B). Seeds from the modified *Ospsy1(I+II)$_{pro}$G* gave the second-highest GUS expression in the endosperm which was restricted in the peripheral region whereas GUS activity was absent in the embryo (G). Very weak GUS expression was also found in the peripheral region of endosperm in seeds of *35S$_{pro}$mG* (D) and *Ospsy1(II)$_{pro}$G*. (F). Samples from the rest of the constructs gave no GUS signals in the endosperm (C) and (E).

When observed through high magnification, as presented in Figure 4.40, more details in the stained tissues could be observed but overall consistent with the results from low magnification. *35S$_{pro}$* directed the strongest GUS expression in the seed coat, aleurone layer and central region of endosperm (B); while *Ospsy1(I+II)$_{pro}$G* directed GUS expression in seed coat, aleurone layer and subaleuroen region of endosperm (G). *Ospsy1(II)$_{pro}$* caused very weak GUS expression in endosperm (F), in additions to seed coat and aleurone layer observed in *Ospsy1$_{pro}$* (E) while only weak GUS expression in endosperm was observed in *35S$_{pro}$mG* (D).

Seed coat (Tegmen)



Embryo and endosperm

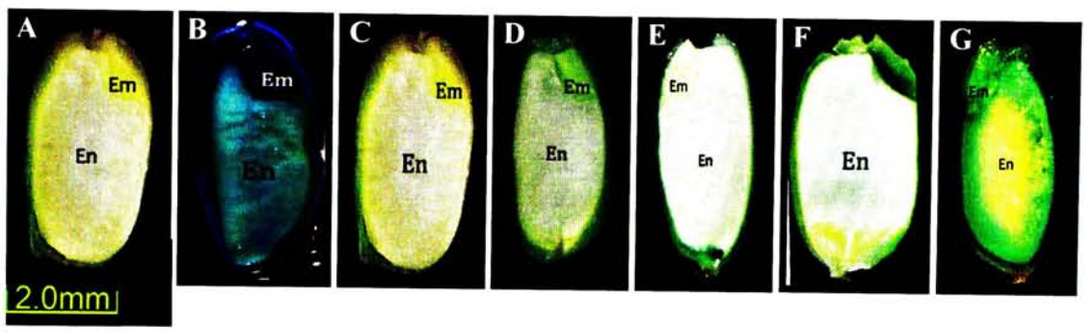


Figure 4.39 Histochemical staining of GUS activity in immature seeds from wild type and transgenic rice for promoter analysis under low magnification (10X)

Immature seeds from wild type and transgenic *Japonica* 9983 rice were collected and dissected transversally. Then they were subjected to histochemical staining for 24 hours. Stained tissues were washed with 70% ethanol overnight. Blue foci developed in seed coat **(upper panel)**, embryo and endosperm **(lower panel)** were observed under light microscope. **(A)** Wild type, **(B)** $35S_{pro}$/GUS, **(C)** $35S_{pro}m$/GUS, **(D)** $35S_{pro}mG$/GUS, **(E)** $Ospsy1_{pro}$/GUS, **(F)** $Ospsy1(II)_{pro}G$/GUS and **(G)** $Ospsy1(I+II)_{pro}G$/GUS. Labels: En, endosperm; and Em, embryo.

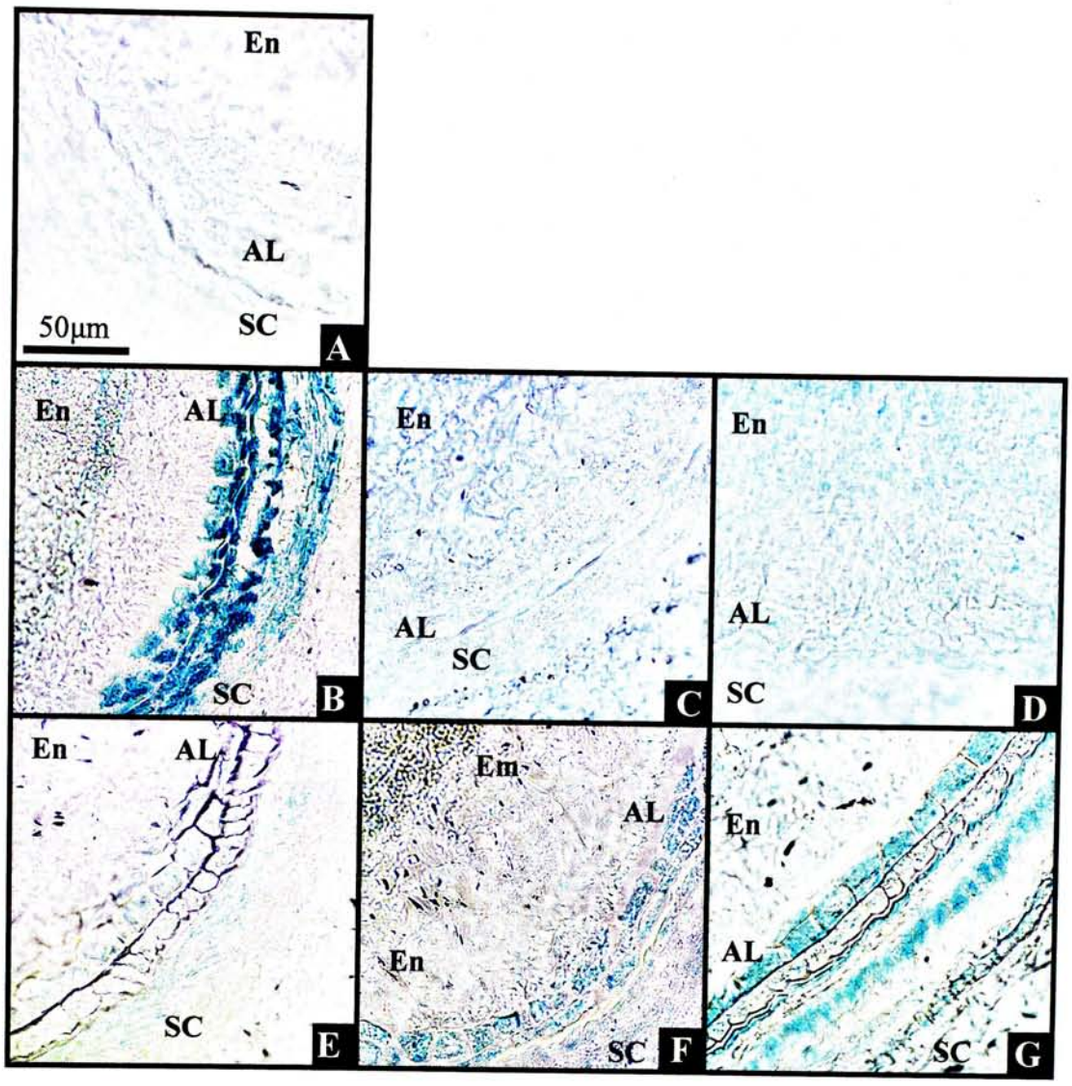


Figure 4.40 Histochemical staining of GUS activity in immature seeds from wild type and transgenic rice for promoter analysis under high magnification (400X)

Immature seeds from wild type and transgenic *Japonica* 9983 rice were collected and dissected transversally. Then they were subjected to histochemical staining for 24 hours. Stained tissues were washed with 70% ethanol overnight and later fixed in paraffin wax (McCormic Scientific).Tissues were sectioned and blue foci developed were observed under fluorescence microscope. **(A)** Wild type, **(B)** $35S_{pro}$/GUS, **(C)** $35S_{pro}m$/GUS, **(D)** $35S_{pro}mG$/GUS, **(E)** $Ospsy1_{pro}$/GUS, **(F)** $Ospsy1(II)_{pro}G$/GUS and **(G)** $Ospsy1(I+II)_{pro}G$/GUS. Labels: SC, seed coat; AL, aleurone layer; En, endosperm; and Em, embryo.

4.2.4.2.2 GUS activity assay

Assay of GUS activity in the endosperm of immature seeds was performed following the same procedures employed for leaves samples. GUS activity detected in the seed samples was presented in Figure 4.41, and the details were summarized in Table 4.7.

The GUS activity detected in the wild type seeds was considered as the background level. Consistent with the histochemical staining results, seeds from $35S_{pro}m$, $35S_{pro}mG$, $Ospsy1_{pro}$ and $Ospsy1(II)_{pro}G$ showed very weak GUS activity which was close to the background level. *p*-values of GUS activity driven by these promoters were greater than 0.05 (*p*-value: 0.87, 0.99, 0.40 and 0.55 respectively), meaning that they showed no statistically significant GUS activity when compared to the wild type.

In contrast, stronger GUS activity was detected in the endosperm from $35S_{pro}$ and $Ospsy1(I+II)_{pro}G$ plants, with *p*-value lower than 0.05, 0.003 and 0.001 respectively. These suggests that the GUS activity directed by these promoters were statistically significant.

When comparing the GUS activity directed by $Ospsy1_{pro}$ with the modified $Ospsy1(I+II)_{pro}G$, the introduction of seven GCN4 had successfully introduce the seed expression activity into the leaf-specific $Ospsy1_{pro}$ (*p*-value: 0.001).

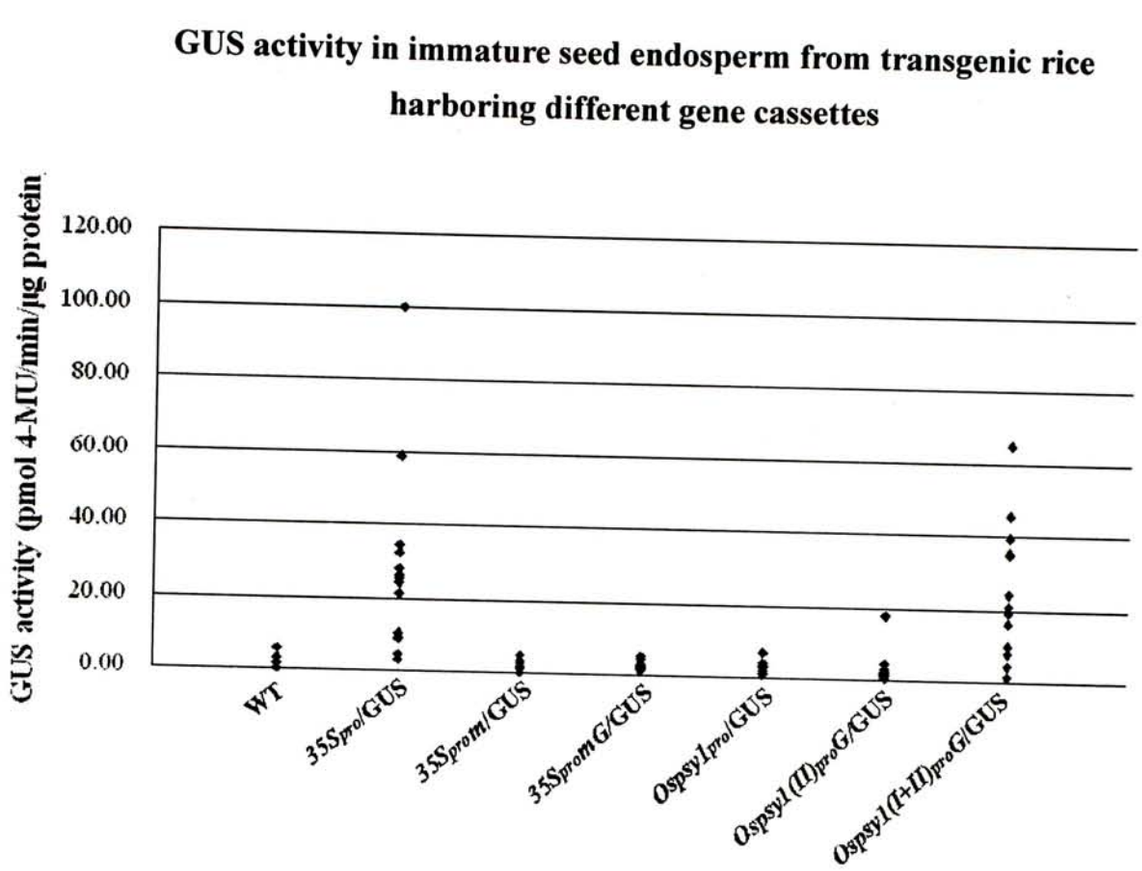


Figure 4.41 GUS activity of total protein from immature seeds of individual plants harboring different constructs

Each point represents the average GUS activity detected for a single seeds sample with three technical repeats. *p*-values of GUS activity compared with wild type (WT) were 0.003, 0.87, 0.99, 0.40, 0.55 and 0.001 respectively.

Table 4.7 Summary of GUS activity detected in immature seeds harboring different gene constructs

Constructs	Plants	Copy number of transgene	GUS activity (pmol 4-MU/min/μg protein)	GUS activity per copy number (pmol 4-MU/min/μg protein)	Standard deviation
Wild type	W-01		3.05		
	W-02		3.01		
	W-03		1.39		
	W-04		5.52		
	W-05		0.00		
	W-06		1.45		1.91
$35S_{pro}$:GUS	2-1	3	78.55	26.18	
	5-1	1	58.80	58.80	
	11-1	2	41.92	20.96	
	32-2	1	10.53	10.53	
	32-3	1	27.79	27.79	
	34-1	1	25.49	25.49	
	35-1	1	99.98	99.98	
	36-1	1	34.31	34.31	
	38-1	1	2.90	2.90	
	38-2	1	8.98	8.98	
	39-1	2	8.65	4.33	
	56-1	1	8.39	8.39	
	82-1	1	23.94	23.94	
	82-2	1	32.16	32.16	25.57
$35S_{pro}m$:GUS	7-1	1	2.48	2.48	
	13-1	1	1.84	1.84	
	15-2	2	3.60	1.80	
	22-1	2	2.34	1.17	
	26-1	1	2.63	2.63	
	43-1	2	1.56	0.78	
	46-1	2	1.52	0.76	
	49-2	1	3.39	3.39	
	50-1	2	1.70	0.85	
	50-2	3	3.38	1.13	
	50-3	1	3.45	3.45	
	60-1	1	3.36	3.36	
	61-1	1	3.27	3.27	
	66-3	1	3.46	3.46	
	71-1	1	4.63	4.63	
	75-2	1	3.25	3.25	
	79-3	2	0.36	0.18	1.29
$35S_{pro}mG$:GUS	11-2	2	3.32	1.66	
	15-1	1	2.57	2.57	
	15-2	1	5.26	5.26	
	23-1	2	4.54	2.27	
	28-2	3	3.49	1.16	
	42-1	4	4.64	1.16	1.30
$35S_{pro}mG$:GUS	45-1	2	2.53	1.27	
	54-1	2	3.16	1.58	
	54-2	2	2.39	1.20	
	64-2	1	3.12	3.12	
	64-4	1	3.01	3.01	
	65-2	1	2.65	2.65	
	66-1	1	4.42	4.42	1.30
$Ospsy1_{pro}$:GUS	1-1	4	3.77	0.94	
	8-1	1	2.82	2.82	
	8-2	1	3.01	3.01	
	9-4	1	3.28	3.28	
	18-1	1	3.77	3.77	
	27-1	2	3.09	1.55	
	46-2	2	7.11	3.56	
	50-2	1	2.94	2.94	
	50-3	2	7.10	3.55	
	61-1	1	6.67	6.67	1.52
$Ospsy1(II)_{pro}G$:GUS	21-3	1	4.42	4.42	
	29-1	2	2.96	1.48	
	30-2	3	2.10	0.70	
	38-1	1	0.76	0.76	
	38-2	1	2.24	2.24	
	44-1	1	17.65	17.65	
	53-1	1	4.25	4.25	
	53-2	2	6.00	3.00	
	53-3	2	2.64	1.32	
	69-1	1	1.64	1.64	
	92-2	4	1.39	0.35	4.91
$Ospsy1(I+II)_{pro}G$:GUS	5-1	1	35.05	35.05	
	9-1	1	45.58	45.58	
	19-1	2	79.04	39.52	
	17-2	3	22.58	7.53	
	22-1	1	9.93	9.93	
	27-1	2	8.68	4.34	
	28-2	2	32.08	16.04	
	37-1	1	1.47	1.47	
	37-2	1	1.33	1.33	
	47-1	1	35.47	35.47	
	47-3	1	7.64	7.64	
	63-1	1	24.25	24.25	
	71-2	1	20.75	20.75	
	78-1	1	19.04	19.04	
	78-2	1	64.80	64.80	18.55

Remarks: GUS activity calculated with negative value was marked in red in blanket.

Chapter 5: Discussion

5.1 Tissue-specificity and endosperm specific expression of rice *psy1* and *psy2*

5.1.1 OsPSY1 and OsPSY2 activities in rice calli

In this project, the constitutive expression of *Ospsy1* and *Ospsy2* under the regulation of *CaMV35S* promoter turned the originally yellow rice calli orange. Western blot analysis revealed higher OsPSY1 and OsPSY2 levels in the transgenic orange calli then in the yellow EMV calli. UPLC analysis revealed increased phytoene and β-carotene contents in the orange calli. The average phytoene contents in *Ospsy1* and *Ospsy2* calli were 5042.3ng/mg and 3499.8ng/mg of dried rice calli, amounting to 755.9% and 494.1% increase respectively. β-carotene, which could not be detected in EMV calli, was found to accumulate at an average amount of 1474.5ng/mg and 792.7ng/mg of dried rice calli in *Ospsy1* and *Ospsy2* calli respectively. All these suggested that both OsPSY1 and OsPSY2 are functional in rice calli and the activity of OsPSY1 is stronger than that of OsPSY2.

Since only phytoene was detected by UPLC analysis, and there was endogenous OsPSY activity in EMV calli, it is expected that the carotenogenic reaction stopped at phytoene. Formation of β-carotene in the rice calli would suggest the presence of functional endogenous carotenogenic enzymes such as phytoene desaturase, ζ-carotene

desaturase, carotene isomerase and lycopen-β-cyclase. The absence of downstream carotenoids in the calli by our study may be due to the rate-limiting OsPSY activity in EMV calli and/or deficiency in certain endogenous carotenoigenic enzymes, thus, the amounts of downstream carotenoids were too low to be detected by UPLC analysis or absent for their detection.

5.1.2 OsPSY1 and OsPSY2 activities in rice leaves

The activities and effects brought forth between overexpression of OsPSY1 and OsPSY2 in rice leaves were very different. The constitutive expression of *Ospsy1* resulted in an unexpected reduction in carotenoids level by 78.8% for β-carotene, 32.2% for xanthophylls and 14% for violaxanthin. Similar scenario has been reported when an endogenous fruit-specific *psy* was constitutively expressed under *CaMV35S* promoter in tomato, leading to a reduction in, even a virtual absence of, carotenoids in transgenic fruits, showing phenotype similar to *psy1*-antisense fruits. Gene silencing or co-suppression was suggested as the reason for this phenomenon (Bramley, 1997). Therefore, this leads us to believe that the severe reduction observed in rice leaves as we observed is due to gene silencing effect exerted by overexpression of *Ospsy1*.

In contrast, constitutive expression of *Ospsy2* led to an increase in carotenoids level by 322.5% for β-carotene, 36.8% for xanthophylls and 16.2% for violaxanthin. This demonstrates the leaf-specific function of *Ospsy2*. The western blot data of *Ospsy2*

leaves correlate well with the increase in carotenoids as detected by UPLC, in that the transgenic leaves contained more OsPSY2 and accumulated more β-carotene, for instance, in transgenic lines 8-1 (76.8%), 89-2 (11.0%), 93-2 (664.6%), 96-2 (135.3%) and 96-3 (211.8%) for $35S_{pro}$/*Ospsy2*; and 70-1 (198.1%), 70-2 (377.5%), 70-3 (11.8%) and 79-2 (155.4%) for Gt-1_{pro}/*Ospsy2*.

5.1.3 OsPSY1 and OsPSY2 activities in rice seeds

Analysis performed on *Ospsy1* and *Ospsy2* transgenic rice demonstrated that both the constitutive expression driven by *CaMV35S* promoter and the endosperm-specific expression driven by Gt-1 promoter of the two *Ospsy* genes resulted in phytoene enhancement, but to different extents. Seed phytoene content was increased by 140.1% to an average of 33.7ng/mg of dry rice endosperm and by 145.7% to 34.4ng/mg of dry rice endosperm under the expression of *Ospsy1* by *CaMV35S* promoter and Gt-1 promoter respectively. As for *Ospsy2*, phytoene content was increased only by 32.1% to an average of 18.5ng/mg of dry rice endosperm and by 42.1% to 19.9ng/mg of dry rice endosperm under the control of *CaMV35S* promoter and Gt-1 promoter respectively. This demonstrates that the activity of OsPSY1 was higher than that of OsPSY2 in rice endosperm and was responsible for carotenoids accumulation in rice seed. This suggests that rice endosperm provides an environment which fits the activity of OsPSY1.

Western blot results on *Ospsy1* were consistent with the phytoene enhancement

data. Seeds with higher OsPSY1 level showed increased phytoene content, for instance, transgenic lines 9-2 (117.0%), 20-2 (132.9%) and 105-1 (141.4%) for $35S_{pro}$/*Ospsy1* and 18-3 (96.4%), 54-1 (120.1%) and 95-2 (202.5%) for Gt-1_{pro}/*Ospsy1*. Variations in phytoene contents among seeds from different transgenic lines transformed with the same gene cassette were most likely due to positional effect of transgene insertion. Interestingly, there were several samples showing increased phytoene contents yet did not show more OsPSY in western blot analysis, such as transgenic lines 5-1 and 105-2 for $35S_{pro}$/*Ospsy1* and 54-1 for Gt-1_{pro}/*Ospsy1*. Similar results have been reported by Burkhardt *et. al.* (1997) that an increase in carotenoids was demonstrated in the absence of immunologically detectable gene products when daffodil *psy* was expressed in rice endosperm. It was suggested that even present in extremely low amounts, carotenogenic enzymes from higher plants are still efficient and suffice for carotenoids accumulation.

As for *Ospsy2*, even its transcript was detected in immature transgenic seeds as shown in northern blot, there was no increased level of OsPSY2. It may be possible that the turnover rate of OsPSY2 in seeds is high, or even being translated into OsPSY2, the protein cannot associate with suitable membrane structure, hence cannot be stored and accumulated in the immature seeds. Further analysis on the localization of OsPSY2 in rice seeds may help to answer this question. Signals with higher molecular weight were detected in transgenic seeds where *Ospsy2* was expressed seed-specifically under the

control of Gt-1 promoter. These unexpected bands may result from the non-specific binding of probe to the RNA of other seed-expressing genes or due to the presence of aberrant *Ospsy2* mRNA. Further analysis is needed to verify the identity of these RNA detected.

To sum up, *Ospsy1* is a suitable candidate for making Golden Rice due to its higher and enhanced enzymatic activity in rice endosperm. Although both the seed-specific Gt-1 promoter and constitutive *CaMV35S* promoter can direct *Ospsy1* expression and promote phytoene generation in seeds, as demonstrated in the transgenic leaves, it was speculated that the constitutive expression of *Ospsy1* may lead to gene silencing and reduce carotenoids contents in leaves. Since carotenoids play important roles in photoprotection of the photosynthetic apparatus in leaves, reduction in carotenoids level may lead to undesirable effects. Thus, a seed-specific promoter should be employed to drive *Ospsy1* in making Golden Rice.

5.2 Analysis of modified rice *psy1* promoter

To study the possibility of adding seed expression activity to the leaf-specific *Ospsy1* promoter by introducing of seed-specific *cis*-acting regulatory element, the GCN4 motif was chosen as it is an essential regulatory element conferring rice endosperm-specific expression.

The modified *Ospsy1* promoter, *Ospsy1(I+II)$_{pro}$G*, with seven GCN4 motifs within introduced 200bp upstream of the TATA box of *Ospsy1*, was able to direct GUS expression in the rice seed endosperm as demonstrated by both histochemical staining and activity assay of the GUS reporter. The expression pattern directed by the modified promoter was similar to that of the rice glutelin promoters, that is, with restricted expression in the aleurone, subaleurone and the peripheral region of rice endosperm. On the other hand, histochemical staining and activity assay of the GUS in transgenic leaves revealed that the strength and the leaf-specific activity of the *Ospsy1* promoter were not affected by the introduction of GCN4 multimer.

This discovery sets light on a new way to direct endosperm-specific expression in rice and to direct tissue- or organ-specific expression of transgenes in other crops for improvement. Thus, instead of using a new promoter, GCN4 can be added to the promoter of a target gene to driven its expression in seeds. The strength of expression could be adjusted by controlling the copy number of GCN4 introduced to the promoter. In addition, further study can be carried out on the transcriptional activator of GCN4, such as RISBZI, to elucidate possible mechanism to adjust the GCN4-harhoring promoter activity in rice. Similar approach can also be employed to broaden this idea to general crop improvement, for example is wheat, maize and barley where GCN4 transcriptional activators have been identified.

5.3 Future prospects of Golden Rice

The focus of this project has been emphasized on increasing the production of β-carotene in rice endosperm and the possibility of using rice homologous genes in the engineering. In the present Golden Rice 2, the high β-carotene accumulation in rice seed was achieved by expressing a maize *psy* gene and a bacterial desaturase gene in the endosperm. Such approach would lead to a mixture of genes from different species; and the use of a bacterial gene for making staple crop may arouse consumer concern on the safety issue of genetically-modified food. In the present study we have successfully demonstrated the possibility of using rice *psy* genes, especially *Ospsy1*, for improving the provitamin A content in rice seed. This can prevent mixing of genes from different species, thus a better social acceptance. Also, it would be easier to determine the allergenicity of Golden Rice, as all of the genes and promoters used are originally from rice's own genome. Our study proves the feasibility of using *Ospsy1* for developing Golden Rice.

As we have already demonstrated the production of phytoene in rice endosperm with the use of rice *psy* genes; the next step is to elucidate the use of other downstream rice carotenogenic enzymes such as the PDS, ZDS, CrtISO and ZISO, so that a complete β-carotene biosynthetic pathway can be engineered into rice endosperm. This would help assessing the possibility of making a new version of Gold Rice solely with

the use of rice's own gene instead of the maize and bacterial copies.

On the other hand, to further enhance the pro-vitamin A content, other approaches and aspects have to be considered, such as the prevention of β-carotene degradation. As reported in early study, the colour of Golden Rice faded upon storage, indicating a reduction in β-carotene due to degradation by light and oxidation. As rice endosperm does not store and accumulate carotenoids normally, there is no carotenoid sequestration mechanism offering protection to β-carotene formed in Golden Rice. In the future, further study can be targeted on the prevention of carotenoids degradation. This can be achieved by elucidating the actions of carotenoids cleaving enzymes or the functions of carotenoid-associating proteins in rice.

Early study focused on plant carotenoid-associated proteins confirmed that these proteins can provide a metabolic sink for carotenoid sequestration in plastids. The fruit-specific overexpression of a pepper carotenoid-binding protein, fibril, in tomato resulted in an increase in carotenoids and carotenoid-derived flavor volatiles (Giuliano *et. al.*, 2008). This suggests a possible way to protect carotenoids from oxygen and light attacks with the use of carotenoid-binding protein in Golden Rice.

Chapter 6: Conclusions

In this project, the enzymatic activities of the two rice PSYs, OsPSY1 and OsPSY2, have been studied in rice calli, leaves and seeds. And the potential of using seed-specific *cis*-acting regulatory element, GCN4, to introduce seed expression activity of the leaf-specific *Ospsy1* promoter has been explored.

For rice calli, the un-organized and undifferentiated cell masses, both OsPSY1 and OsPSY2 were functional. Constitutive expression of their two genes resulted in accumulation of phytoene to 5042.3ng/mg and 3499.8ng/mg of dried rice calli, and β-carotene to 1474.5ng/mg and 792.7ng/mg of dried rice calli respectively.

For leaf, constitutive expression of *Ospsy1* led to an undesirable gene silencing effect causing severe reduction in leaf carotenoids. In contrast, overexpression of *Ospsy2* resulted in an increase of β-carotene by 322.5% and significant increases in other carotenoid species, indicating the leaves-specific activity of *Ospsy2*.

In order to determine the potential of using *Ospsy1* and *Ospsy2* for making Golden Rice, they were expressed in a seed-specific manner. Although both of them could lead to phytoene enhancement, the enzymatic activity of OsPSY1 was much stronger, resulting with an increase by 145.7%, 34.4ng/mg of dry rice endosperm. This suggests the activity of OsPSY1 is responsible for carotenoids accumulation in rice seed and therefore, is a suitable candidate for making Golden Rice.

Finally, this study has successfully demonstrated the introduction of seed-specific *cis*-acting regulatory element, GCN4 multimer, with a total of seven GCN4 copies, into the *Ospsy1* promoter can confer seed expression activity without affecting the leaf-specific activity.

References

1 Al-Babili, S. and Beyer, P. 2005. Golden Rice - five years on the road - five years to go? *Trends Plant Sci.* **10**, 565-573.

2 Al-Babili, S., Hoa, T. T. C. and Schaub, P. 2006. Exploring the potential of the bacterial carotene desaturase CrtI to increase the β-carotene content in Golden Rice. *J. Exp. Bot.* **57**, 1007-1014.

3 Al-Babili, S., Hugueney, P., Scheldz, M., Welsch, R., Frohnmeyer, H. and Laule, O. 2000. Identification of a novel gene coding for neoxanthin synthase from *Solanum tuberosum*. *FEBS Lett.* **485**, 168-172.

4 Al-Babili, S., von Lintig, J., Haubruck, H. and Beyer, P. 1996. A novel soluble form of phytoene desaturase in *Narcissus pseudonarcissus* is Hsp 70-complexed and competent for flavinylation, membrane association and enzymatic activation. *Plant J.* **9**, 601-612.

5 Balmer, J. E. and Blomhoff, R. 2002. Gene expression regulation by retinoic acid. *J. Lipid Res.* **43**, 1773-1808.

6 Banajeh, S. M. 2003. Is 12-monthly vitamin A supplementation of preschool children effective? An observational study of motality rates for severe dehydrating diarrhea in Yemen. *S. Afr. J. Clin. Nutr.* **16**, 137-142.

7 Barber, N. 2003. The tomato: an important part of the urologist's diet? *BJU. Intnal.* **91**, 307-309.

8 Bartley, G. E. and Scolnik, P. A. 1993. cDNA cloning, expression during development, and genome mapping of *psy2*, a second tomato gene encoding phytoene synthase. *J. Biol. Chem.* **268**, 25718-25721.

9 Bartley, G. E. and Scolnik, P. A. 1995. Plant carotenoids: pigments for photoprotection, visual attraction, and human health. *Plant Cell.* **7**, 1027-1038.

10 Bewley, J. D. 1997. Seed germination and dormancy. *Plant Cell.* **9**, 1055-1066.

11 Beyer, P., Al-Babili, S., Ye, X., Lucca, P., Schaub, P., Welsch, R. and Potrykus, I. 2002. Golden Rice: introducing the β-carotene biosynthesis pathway into rice endosperm by genetic engineering to defeat vitamin A deficiency. *J. Nutr.* **132**, 506S-510S.

12 Beyer, P., Kroncke, U. and Nievelstein, V. 1991. On the mechanism of the lycopene isomerase cyclase reaction in *Narcissus pseudonarcissus* L chromoplasts. *J. Biol. Chem.* **266**, 17072-17078.

13 Beyer, P., Weiss, G. and Kleinig, H. 1985. Solubilization and reconstitution of the membrane bound carotenogenic enzymes from daffodil chromoplasts. *Eur. J. Biochem.* **153**, 341-346.

14 Blomhoff, R. and Blomhoff, H. K. 2006. Overview of retinoid metabolism and function. *J. Neurobiol.* **66**, 606-630.

15 Bonk, M., Hoffmann, B., von Lintig, J., Schledz, M., Al-Babili, S., Hobeika, E., Kleinig, H. and Beyer, P. 1997. Chloroplast import of four carotenoid biosynthetic enzymes in vitro reveals differential fates prior to membrane binding and oligomeric assembly. *Eur. J. Biochem.* **247**, 942-950.

16 Bouvier, F., d'Harlingue, A., Backhaus, R. A., Kumagai, M. H. and Camara, B. 2000. Identification of neoxanthin synthase as a carotenoid cyclase paralog. *Eur. J. Biochem.* **267**, 6346-6352.

17 Bramley, P. M. 1997. The regulation and genetic manipulation of carotenoid biosynthesis in tomato fruit. *Pure & Appl. Chem.* **69**, 2159-2162.

18 Breitenbach, J., Kuntz, M., Takaichi, S. and Sandmann, G. 1999. Catalytic properties of an expressed and purified higher plant type zeta-carotene desaturase from *Capsicum annuum*. *Eur. J. Biochem.* **265**, 376-383.

19 Brown, L., Rimm, E. B., Seddon, J. M., Giovannucci, E. L., Chasan-Taber, L. and Spiegelman, D. 1999. A prospective study of carotenoid intake and risk of cataract extraction in US men. *Am. J. Clin. Nutr.* **70**, 517-524.

20 Buckner, B., San Miguel, P., Janick-Buckner, D. and Bennetzen, J. L. 1996. The *y1* gene of maize codes for phytoene synthase. *Genet.* **143**, 479-488.

21 Burkhardt, P. K., Beyer, P., Wunn, J., Kloti, A., Armstrong, G. A., Schledz, M., von Lintig, J. and Potrykus, I. 1997. Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. *Plant J.* **11**, 1071-1078.

22 Busch, M., Seuter, A. and Hain, R. 2002. Functional analysis of the early steps of carotenoid biosynthesis in tobacco. *Plant Physiol.* **128**, 439-453.

23 Bustos, M. M., Begum, D., Kalkan, F. A., Battraw, M. J. and Hall, T. C. 1991. Positive and negative *cis*-acting DNA domains are required for spatial and temporal regulation of gene expression by a seed storage protein promoter. *EMBO J.* **10**, 1469-1479.

24 Camara, B., Bardat, F. and Moneger, R. 1982. Sites of biosynthesis of carotenoids in *Capsicum* chromoplasts. *Eur. J. Biochem.* **127**, 255-258.

25 Camara, B. and Dogbo, O. 1986. Demonstration and solubilization of lycopene cyclase from *Capsicum* chromoplast membranes. *Plant Physiol.* **80**, 172-174.

26 Castenmiller, J. M. and West, C. E. 1998. Bioavailability and bioconversion of carotenoids. *Annu. Rev. Nutr.* **18**, 19-38.

27 Chen. L., Stacewicz-Sapuntzakis, M., Duncan, C., Sharifi, R., Ghosh, L. and van Breemen, R. 2001. Oxidative DNA damage in prostate cancer patients consuming tomato sauce-based entrees as a whole-food intervention. *J. Natl. Cancer Inst.* **93**, 1872-1872.

28 Christian, P., West, K. P., Khatry, S. K., Katz, J., Shrestha, S. R. and Pradhan, E. K. 1998. Night blindness of pregnancy in rural Nepal – nutritional and health risks. *Int. J. Epidemiol.* **27**, 231-237.

29 Chu, C. C. 1978. The N6 medium and its applications to anther culture of cereal crops. In: *Proceedings of symposium on plant tissue culture.* Science press, Peking.

30 Collins, A. R. 2001. Carotenoids and genomic stability. *Mutat. Res.* **475**, 21-28.

31 Cowan, A. K. and Richardson, G. R. 1993. The biosynthesis of abscisic acid from *all-trans*-β-carotene in a cell-free system from *Citrus sinensis* exocarp. *Plant Cell Physiol.* **34**, 969-972.

32 Croce, R., Muller, M. G., Bassi, R. and Holzwarth, A. R. 2001. Carotenoid-to-chlorophyll energy transfer in recombinant major light-harvesting complex (LHCII) of higher plants. I. femtosecond transient absorption measurements. *Biophysical J.* **80**, 901-915.

33 Cunningham, F. S. and Gantt, E. 1998. Genes and enzymes of carotenoid biosynthesis in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 557-583.

34 Datta, K., Baisakh, N., Oliva, N., Torrizo, L., Abrigo, E., Tan, J., Rai, M., Rehana, S., Al-Babili, S., Beyer, P., Potrykus, I. and Datta, S. K. 2003. Bioengineered 'golden' indica rice cultivars with β-carotene metabolism in the endosperm with hygromycin and mannose selection systems. *Plant Biotech. J.* **1**, 81-90.

35 Davuluri, G. R., van Tuinen, A., Mustilli, A. C., Manfredonia, A., Newman, R., Burgess, D., Brummell, D. A., King, S. R., Palys, J., Uhlig, J., Pennings, H. M. and Bowler, C. 2004. Manipulation of DET1 expression in tomato results in photomorphogenic phenotypes caused by post-transcriptional gene silencing. *Plant J.* **40**, 344-354.

36 DellaPenna, D. and Pogson, B. J. 2006. Vitamin synthesis in plants: tocopherols and carotenoids. *Annu. Rev. Plant. Biol.* **57**, 711-738.

37 Demmig-Adams, B. and Adams, W. W. 1996. The role of xanthophylls cycle carotenoids in the protection of photosynthesis. *Trends Plant Sci.* **1**, 21-26.

38 Deruere, J., Romer, S., d'Harlingue, A., Backhaus, R. A., Kuntz, M. and Camara, B. 1994. Fibril assembly and carotenoid overaccumulation in chromoplasts: a model for supramolecular lipoprotein structures. *Plant Cell.* **6**, 119-133.

39 Dogbo, O. and Camara, B. 1987. Purification of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthase from *Capsicum* chromoplasts by affinity chromatography. *Biochem. Biophys. Acta.* **920**, 140-148.

40 Dogbo, O., Laferriere, A., d'Harlingue, A. and Camara, B. 1988. Carotenoid biosynthesis: isolation and characterization of a bifunctional enzyme catalyzing the synthesis of phytoene. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7054-7058.

41 Ducreux, L. J. M., Morris, W. L., Hedley, P. E., Shepherd, T., Davies, H. V., Millam, S. and Taylor, M. A. 2004. Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. *J. Exp. Bot.* **56**, 81-89.

42 Eichler, O., Sies, H. and Stahl, W. 2002. Divergent optimum levels of lycopene, beta-carotene and lutein protecting against UVB irradiation in human fibroblasts. *Photochem. Photbiol.* **75**, 503-506.

43 El-Agamey, A., Lowe, G. M., McGarvey, D. J., Mortensen, A., Phillip, D. M., Truscott, T. G. and Young, A. J. 2004. Carotenoid radical chemistry and antioxidant/pro-oxidant properties. *Arch. Biochem. Biophys.* **430**, 37-48.

44 Frank, H. A., Chynwat, V., Desamero, R. Z. B., Farhoosh, R., Erickson, J. and Bautista, J. 1997. On the photophysics and photochemical properties of carotenoids and their role as light-harvesting pigments in photosynthesis. *Pure & Appl. Chem.* **69**, 2117-2124.

45 Fraser, P. D. and Bramley, P. M. 2004. The biosynthesis and nutritional uses of carotenoids. *Prog. Lipid Res.* **43**, 228-265.

46 Fraser, P. D., Kiano, J. W., Truesdale, M. R., Schuch, W. and Bramley, P. M. 1999. Phytoene synthase-2 enzyme activity in tomato does not contribute to carotenoid synthesis in ripening fruit. *Plant Mol. Biol.* **40**, 687-698.

47 Fraser, P. D., Romer, S., Shipton, C. A., Mills, P. B., Kiano, J. W., Misawa, N., Drake, R. G., Schuch, W. and Bramley, P. M. 2002. Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. *PNAS.* **99**, 1092-1097.

48 Fraser, P. D., Schuch, W. and Bramley, P. M. 2000. Phytoene synthase from tomato (*Lycopersicon escudentuim*) chloroplasts-partial purification and biochemical properties. *Planta.* **211**, 361-369.

49 Fraser, P. D., Truesdale, M. R., Bird, C. R., Schuch, W. and Bramley, P. M. 1994. Carotenoid biosynthesis during tomato fruit development (evidence for tissue-specific gene expression). *Plant Physiol.* **105**, 405-413.

50 Fray, R. G. and Grierson, D. 1993. Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation and co-suppression. *Plant Mol. Biol.* **22**, 589-602.

51 Fray, R. G., Wallace, A., Fraser, P. D., Valero, D., Hedden, P., Bramley, P. M. and Grierson, D. 1995. Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. *Plant J.* **8**, 693-701.

52 Gallagher, C. E., Matthews, P. D., Li, F. and Wurtzel, E. T. 2004. Gene duplication in the carotenoid biosynthetic pathway preceded evolution of the grasses. *Plant Physiol.* **135**, 1776-1783.

53 Galpaz, N., Ronen, G., Khalfa, Z., Zamir, D. and Hirschberg, J. 2006. A chromoplast-specific carotenoid biosynthesis pathway is revealed by cloning of the tomato *white-flower* locus. *Plant Cell.* **18**, 1947-1960.

54 Giliberto, L., Perrotta, G., Pallara, P., Weller, J. L., Fraser, P. D., Bramley, P. M., Fiore, A., Tavazza, M. and Giuliano, G. 2005. Manipulation of the blue light photoreceptor cryptochrome 2 in tomato affects vegetative development, flowering time, and fruit antioxidant content. *Plant Physiol.* **137**, 199-108.

55 Giorio, G., Stigliani, A. L. and D'Ambrosio, C. 2008. Phytoene synthase genes in tomato (*Solanum lycopersicum* L.) – new data on the structures, the deduced amino acid sequences and the expression patterns. *FEBS J.* **275**, 527-535.

56 Giovannucci, E., Atscherio, A. and Rimm, E. B. 1995. Intake of carotenoids and retinol in relation to risk of prostate cancer. *J. Natl. Cancer Inst.* **87**, 1767-1776.

57 Giuliano, G., Aquilani, R. and Dharmapuri, S. 2000. Metabolic engineering of plant carotenoids. *Trends Plant Sci.* **10**, 406-409.

58 Giuliano, G., Bartley, G. E. and Scolnik, P. A. 1993. Regulation of carotenoid biosynthesis during tomato development. *Plant Cell.* **5**, 379-387.

59 Giuliano, G., Tavazza, R., Diretto, G., Beyer, P. and Taylor, M. A. 2008. Metabolic engineering of carotenoid biosynthesis in plants. *Trends in Biotechnol.* **26**, 139-145.

60 Gollnick, P. M., Hopfenmuller, W., Hemmes, C., Chun, S. C., Sundermeier, K. and Biesalski, H. K. 1996. Systemic β-carotene plus topical sunscreen are an optimal protection against harmful effects of natural UV-sunlight: results of the Berlin-Eilath study. *Eur. J. Dermatol.* **6**, 200-205.

61 Groot, S. P. C. and Karssen, C. M. 1992. Dormancy and germination of abscisic acid-deficient tomato seeds. *Plant Physiol.* **99**, 952-958.

62 Hadley, C. W., Clinton, S. K. and Schwartz, S. J. 2003. The consumption of processed tomato products enhances plasma lycopene concentrations in association with a reduced lipoprotein sensitivity to oxidative damage. *J. Nutr.* **133**, 727-732.

63 Han, H., Li, Y. and Zhou, S. 2008. Overexpression of phytoene synthase gene from *Salicornia europaea* alters response to reactive oxygen species under salt stress in transgenic *Arabidopsis*. *Biotechnol. Lett.* **30**, 1501-1507.

64 Havaux, M. and Kloppstech, K. 2001. The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in *Arabidopsis npq* and *tt* mutants. *Planta.* **213**, 953-966.

65 Hirschberg, J. 2001. Carotenoid biosynthesis in flowering plants. *Curr. Opin. Plant Biol.* **4,** 210-218.

66 Horton, P. and Ruban, A. 2004. Molecular design of the photosystem II light-harvesting antenna: photosynthesis and photoprotection. *J. Exp. Bot.* **56**, 365-373.

67 Howitt, C. A. and Pogson, B. J. 2006. Carotenoid accumulation and function in seeds and non-green tissues. *Plant Cell Envir.* **29**, 435-445.

68 Humphrey, J. H., West, K. P. and Sommer, A. 1992. Vitamin A deficiency and attributable mortality among under-5-year-olds. *WHO Bull.* **70**, 225-232.

69 Jackson, M. J. 1997. The assessment of bioavailability of micronutrients: introduction. *Eur. J. Clin. Nutr.* **51**, S1-S2.

70 Janick-Buckner, D., Hammock, J. D., Johnson, J. M., Osborn, J. M. and Buckner, B. 1999. Biochemical and ultrastructural analysis of the *y10* mutant of maize. *J. Hered.* **90**, 507-513.

71 Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. 1987. GUS fusions: beta-glucuronidase as a sensitive versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901-3907.

72 Kasahara, H., Hanada, A., Kuzuyama, T., Takagi, M., Kamiya, Y. and Yamaguchi, S. 2002. Contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthesis of gibberellins in *Arabidopsis*. *J. Biol. Chem.* **277**, 45188-45194.

73 Kiefer, C., Hessel, S., Lampert, J. M., Vogt, K., Lederer, M. O., Breithaupt, D. E. and von Lintig, J. 2002. Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. *J. Biol. Chem.* **276**, 14110-14116.

74 Kim, J. and DellaPenna, D. 2006. Defining the primary route for lutein synthesis in plants: the role of *Arabidopsis* carotenoid β-ring hydroxylase CYP97A3. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 3474-3479.

75 Kirk, J. T. and Tiliney-Bassett, R. A. 1978. Proplastids, etioplasts, amyloplasts, chromoplasts and other plastids. In: *The plastids: Their chemistry, structure, growth and inheritance*. pp 217-239. Elsevier/North Holland Biomedical Press, Amsterdam.

76 Kleinig, H. 1989. The role of plastids in isoprenoid biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 39-59.

77 Kohlmeier, L. and Hastings, S. B. 1995. Epidemiologic evidence of a role of carotenoids in cardiovascular disease prevention. *Am. J. Clin. Nutr.* **62**, 1370S-1376S.

78 Kohlmeier, L., Kark, J. D., Gomez-Garcia, E., Martin, B. C., Steck, S. E., Kardinaal, A. F. M., Ringstad, J., Thamm, M., Masaev, V., Riemersma, R., Martin-Moreno, J. M., Huttunen, J. K. and Kok, F. J. 1997. Lycopene and myocardial infarction risk in EURAMIC study. *Am. J. Epidemiol.* **146**, 618-626.

79 Kuhlbrandt, W. 1994. Structure and function of the plant light-harvesting complex, LH-II. *Curr. Opin. Struct. Biol.* **4**, 519-528.

80 Lamb, T. D. and Pugh, E. N. 2004. Dark adaptation and the retinoid cycle of vision. *Prog. Retinal. Eye. Res.* **23**, 307-380.

81 Lange, B. M. and Ghassemian, M. 2003. Genome organization in *Arabidopsis thaliana*: a survey for genes involved in isoprenoid and chlorophyll metabolism. *Plant Mol. Biol.* **51**, 925-948.

82 Li, F., Murillo, C. and Wurtzel, E. T. 2007. Maize *Y9* encodes a product essential for 15-*cis*-zeta-carotene isomerization. *Plant Physiol.* **144**, 1181-1189.

83 Li, F., Vallabhaneni, R. and Wurtzel, E. T. 2008a. *PSY3*, a new member of the phytoene synthase gene family conserved in the *Poaceae* and regulator of abiotic stress-induced root carotenogenesis. *Plant Physiol.* **146**, 1333-1345.

84 Li, F., Vallabhaneni, R., Yu, J., Rocheford, T. and Wurtzel, E. T. 2008b. The maize phytoene synthase gene family: overlapping roles for carotenogenesis in endosperm, photomorphogenesis, and thermal stress tolerance. *Plant Physiol.* **147**, 1334-1346.

85 van Lieshout, M. and de Pee, S. 2005. Vitamin A equivalency estimates: understanding apparent differences. *Am. J. Clin. Nutr.* **81**, 943-944.

86 Lindgren, L. O., Stalberg, K. G. and Hoglund, A. 2003. Seed-specific overexpression of an endogenous *Arabidopsis* phytoene synthase gene results in delayed germination and increased levels of carotenoids, chlorophyll, and abscisic acid. *Plant Physiol.* **132**, 779-785.

87 Lindqvist, A. and Andersson, S. 2002. Biochemical properties of purified recombinant human beta-carotene 15, 15'-monooxygenase. *J. Biol. Chem.* **277**, 23942-23948.

88 von Lintig, J. and Vogt, K. 2000. Filling the gap in vitamin A research. Molecular identification of an enzyme cleaving beta-carotene to retinal. *J. Biol. Chem.* **275**, 11915-11920.

89 Liu, Y., Gur, A., Ronen, G., Causse, M., Damidaux, R., Buret, M., Hirschberg, J. and Zamir, D. 2003. There is more to tomato fruit colour than candidate carotenoid genes. *Plant Biotechnol. J.* **1**, 195-207.

90 Liu, Y., Roof, S., Ye, Z., Barry, C., van Tuinen, A., Vrebalov, J., Bowler, C. and Giovannoni, J. 2004. Manipulation of light signal transduction as a means of modifying fruit nutritional quality in tomato. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 9897-9902.

91 Lutzow, M. and Beyer, P. 1988. The isopentenyl-diphosphate A-isomerase and its relation to the phytoene synthase complex in daffodil chromoplasts. *Biochem. Biophys. Acta.* **959**, 118-126.

92 Maluf, M. P., Saab, I. N., Wurtzel, E. T. and Sachs, M. M. 1997. The *viviparous12* maize mutant is deficient in abscisic acid, carotenoids, and chlorophyll synthesis. *J. Exp. Bot.* **48**, 1259-1268.

93 Matthew, P. D., Luo, R. and Wurtzel, E. T. 2003. Maize phytoene desaturase and ζ-carotene desaturase catalyse a poly-Z desaturation pathway: implications for genetic engineering of carotenoid content among cereal crops. *J. Exp. Bot.* **54**, 2215-2230.

94 Maudinas, B., Bucholtz, M. L., Papastephanou, C., Katiyar, S. S., Briedis, A. V. and Porter, J. W. 1975. Adenosine 5'-triphosphate stimulation of the activity of a partially purified phytoene synthetase comples. *Biochem. Biophys. Res. Commun.* **66**, 430-436.

95 Munne-Bosch, S. and Alegre, L. 2000. Changes in carotenoids, tocopherols and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in *Rosmarinus officinalis* plants. *Planta*, **210**, 925-931.

96 Nagao, A. 2004. Oxidative conversion of carotenoids to retinoids and other products. *J. Nutr.* **134**, 237S-240S.

97 Nahum, A., Hirsch, K., Danilenko, M., Watts, C. K. W., Prall, O. W. J. and Levy, J. 2001. Lycopene inhibition of cell cycle progression in breast and endometrial cancer cells is associated with reduction in cyclin D levels and retention of $p27^{kip1}$ in the cyclin E-cdk2 complexes. *Oncogene*. **20**, 3428-3436.

98 Naik, P. S., Chanemougasoundharam, A., Khurana, S. M. P. and Kalloo, G. 2003. Genetic manipulation of carotenoid pathway in higher plants. *Curr. Biol.* **85**, 1423-1430.

99 Nakase, M., Aoki, N., Matsuda, T. and Adachi, T. 1997. Characterization of a novel rice bZIP protein which binds to the α-globulin promoter. *Plant Mol. Biol.* **33**, 513-522.

100 Nelis, H. J. and de Leenheer, A. P. 1991. Microbial sources of carotenoid pigments used in foods and feeds. *J. Appl. Bacteriol.* **70**, 181-191.

101 Onodera, Y., Suzuki, A., Wu, C. Y., Washida, H. and Takaiwa, F. 2001. A rice functional transcriptional activator, RISBZ1, responsible for endosperm-specific expression of storage protein genes through GCN4 motif. *J. Biol. Chem.* **276**, 14139-14152.

102 Otten, J. J., Hellwig, J. P. and Meyers, L. D. eds. 2006. Dietary reference intakes: the essential guide to nutrient requirements. pp 170-181. The National Academies Press, Washington, DC.

103 Paine, J. A., Shipton, C. A., Chaggar, S., Howells, R. M., Kennedy, M. J., Vernon, G., Wright, S. Y., Hinchliffe, E., Adams, J. L., Silverstone, A. L. and Drake, R. 2005. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotechnol.* **23**, 482-487.

104 Park, H., Kreunen, S. S., Cuttriss, A. J., DellaPenna, D. and Pogson, B. J. 2002. Identification of the carotenoid isomerase provides insight into carotenoid biosynthesis, prolamella body formation, and photomorphogenesis. *Plant Cell.* **14**, 321-332.

105 Parry, A. D. and Horgan, R. 1992. Abscisic acid biosynthesis in roots. The identification of potential abscisic acid precursors, and other carotenoids. *Planta.* **187**, 185-191.

106 de Pater, S., Katagiri, F., Kijne, J. and Chau, N. H. 1994. bZIP proteins bind to a palin-dromic sequence without an ACGT core located in a seed-specific element of the pea lectin promoter. *Plant J.* **6**, 133-140.

107 de Pee, S. and West, C. E. 1996. Dietary carotenoids and their role in combating vitamin A deficiency: a review of the literature. *Eur. J. Clin. Nutr.* **50**, S38-S53.

108 Pepperberg, D. R. and Crouch, R. K. 2001. An illuminating new step in visual-pigment regeneration. *Lancet.* **358**, 298-299.

109 Qu, L. Q. and Takaiwa, F. 2004. Evaluation of tissue specificity and expression strength of rice seed component gene promoters in transgenic rice. *Plant Biotechnol. J.* **2**, 113-125.

110 Qu, L. Q., Xing, Y. P., Liu, W. X., Xu, X. P. and Song, Y. R. 2008. Expression pattern and activity of six glutelin gene promoters in transgenic rice. *J. Exp. Bot.* **59**, 2417-2424.

111 Redmond, T. M., Gentleman, S., Duncan, T., Yu, S., Wiggert, B. and Gantt, E. 2001. Identification, expression, and substrate specificity of a mammalian beta-carotene 15, 150-dioxygenase. *J. Biol. Chem.* **276**, 6560-6565.

112 Rice, A. L., Stoltzfus, R. J., de Francisco, A. and Kjolhede, C. 2000. Evaluation of serum retinol, the modified-relative-dose-response ratio, and breast-milk vitamin A as indicators of response to postpartum maternal vitamin A supplementation. *Am. J. Clin. Nutr.* **71**, 799-806.

113 Rissler, H. M. and Pogson, B. J. 2001. Antisense inhibition of the beta-carotene hydroxylase enzyme in *Arabidopsis* and the implications for carotenoid accumulation, photoprotection and antenna assembly. *Photosynth. Res.* **67**, 127-137.

114 Ross, D. 2002. Recommendations for vitamin A supplementation. *J. Nutr.* **131**, 2902S-2906S.

115 Sandmann, G. 2001. Genetic manipulation of carotenoid biosynthesis: strategies, problems and achievements. *Trends Plant Sci.* **6**, 14-17.

116 Schaub, P., Al-Babili, S., Drake, R. and Beyer, P. 2005. Why is Golden Rice golden (yellow) instead of red? *Plant Physiol.* **138**, 441-450.

117 Schledz, M., Al-Babili, S., von Lintig, J., Haubruck, H., Rabbani, S., Kleinig, H. and Beyer, P. 1996. Phytoene synthase from *Narcissus pseudonarcissus*: functional expression, galactolipid requirement, topological distribution in chromoplasts and induction during flowering. *Plant J.* **10**, 781-792.

118 Scott, K. J. and Hart, D. J. 1994. The carotenoid composition of vegetables and fruit commonly consumed in the UK. Norwich, IFR.

119 Shewmaker, C. K., Sheehy, J. A., Daley, M., Colburn, S. and Ke, D. Y. 1999. Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J.* **20**, 401-412.

120 Slattery, M. L., Benson, J., Curtin, K., Khe-Ni, M., Schaeffer, D. and Potter, J. D. 2000. Carotenoids and colon cancer. *Am. J. Clin. Nutr.* **71**, 575-582.

121 Solomons, N. W. 2006. Vitamin A. In: *Present knowledge in nutrition.* 9th ed. vol. 1. Bowman, B. A. and Russell, R. M., eds. pp 157-183. International Life Sciences Institute, Washington, DC.

122 Sommerburg, O., Keunen, J. E. E., Bird, A. C. and van Kuijk, F. J. G. M. 1998. Fruits and vegetables that are sources for lutein and zeaxanthin: the macular pigment in hyman eyes. *Br. J. Opthamol.* **82**, 907-910.

123 Stahl, W., Heinrich, U., Jungmann, H., Sies, H. and Tronnier, H. 2000. Carotenoids and carotenoids plus vitamin E protect against ultraviolet light-induced erythema in humans. *Am. J. Clin. Nutr.* **71**, 795-798.

124 Stein, A. J., Sachdev, H. P. S. and Qaim, A. 2008. Genetic engineering for the poor: Golden Rice and public health in India. *World Develop.* **36**, 144-158.

125 Sun, Z., Gantt, E. and Cunningham, F. X. 1996. Cloning and functional analysis of the β-carotene hydroxylase of *Arabidopsis thaliana*. *J. Biol. Chem.* **271**, 24349-24352.

126 Suzuki, A., Wu, C. Y., Washida, H. and Takaiwa, F. 1998. Rice MYB protein OSMYB5 specifically binds to the AACA motif conserved among promoters of genes for storage protein glutelin. *Plant Cell Physiol.* **39**, 555-559.

127 Takaiwa, F., Yamanouchi, U., Yoshihara, T., Washida, H., Tanabe, F., Kato, A. and Yamada, K. 1996. Characterization of common *cis*-regulatory elements responsible for the endosperm-specific expression of members of the rice glutelin multigene family. *Plant Mol. Biol.* **30**, 1207-1221.

128 Talukder, A., Kiess, L., Huq, N., de Pee, S., Darnton-Hill, I. and Bloem, M. W. 2000. Increasing the production and consumption of vitamin A-rich fruits and vegetables: lessons learned in taking the Bangladesh homestead gardening programme to a national scale. *Food Nutr. Bull.* **21**, 165-172.

129 Tang, G., Qin, J., Dolnikowski, G. G., Russell, R. M. and Grusak, M. A. 2009. Golden Rice is an effective source of vitamin A. *Am. J. Clin. Nutr.* **89**, 1776-1783.

130 Taylor, M. and Ramsay, G. 2005. Carotenoid biosynthesis in plant storage organs: recent advances and prospects for improving plant food quality. *Physiol. Plant.* **124**, 143-151.

131 Than, A., Bramley, P. M., Davies, B. H. and Rees, A. F. 1972. Stereochemistry of phytoene. *Phytochem.* **11**, 3187-3190.

132 Toriyama, K. and Hinata, K. 1985. Cell suspension and protoplast culture in rice. *Plant Sci.* **41**, 179-183.

133 Tzamarias, D., Pu, W. T. and Struhl, K. 1992. Mutations in the bZIP domain of yeast GCN4 that alter DNA-binding specificity. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2007-2011.

134 Vicente-Carbajosa, J., Moose, S. P., Parsons, R. L. and Schmidt, R. J. 1997. A maize zinc-finger protein binds the prolamin box in zein gene promoters and interacts with the basic leucine zipper transcriptional activator Opaque2. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7685-7690.

135 Wald, G. 1968. Molecular basis of visual excitation. *Science*. **162**, 230-239.

136 Welsch, R., Beyer, P., Hugueney, P., Kleinig, H. and von Lintig, J. 2000. Regulation and activation of phytoene synthase, a key enzyme in carotenoid biosynthesis, during photomorphogenesis. *Planta*. **211**, 846-854.

137 Welsch, R., Wust, F., Bar, C., Al-Babili, S. and Beyer, P. 2008. A third phytoene synthase is devoted to abiotic stress-induced abscisic acid formation in rice and defines functional diversification of phytoene synthase genes. *Plant Physiol*. **147**, 367-380.

138 West, C. E. and Castenmiller, J. J. 1998. Quantification of the "SLAMENGHI" factors for carotenoid bioavailability and bioconversion. *Inter. J. Vit. Nutr*. **68**, 371-377.

139 West, K. P. 2002. Extent of vitamin A deficiency among preschool children and women of reproductive age. *J. Nutr*. **132**, 2857S-2866S.

140 West, K. P. and Darnton-Hill, I. 2008. Vitamin A deficiency. In: *Nutrition and Health: Nutrition and Health in Developing Countries.* 2nd ed. Semba, R. D. and Bloem, M. W., eds. pp 377-433.Humana Press, Totowa, NJ.

141 West, K. P., Katz, J., Khatry, S. K., LeClerq, S. C., Pradhan, E. K. and Shrestha, S. R. 1999. Double blind, cluster randomized trial of low dose supplementation with vitamin A or β-carotene on mortality related to pregnancy in Nepal. *BMJ*. **318**, 570-575.

142 Wu, C. Y., Adachi, T., Hatano, T., Washida, H., Suzuki, A. and Takaiwa, F. 1998a. Promoters of rice seed storage protein genes direct endosperm-specific gene expression in transgenic rice. *Plant Cell Physiol*. **39**, 885-889.

143 Wu, C. Y., Suzuki, A., Washida, H. and Takaiwa, F. 1998b. The GCN4 motif in a rice glutelin gene is essential for endosperm-specific gene expression and is activated by Opaque-2 in transgenic rice plants. *Plant J*. **14**, 673-683.

144 Wu, C. Y., Washida, H., Onodera, Y., Harada, K. and Takaiwa, F. 2000. Quantitative nature of the prolamin-box, ACGT and AACA motifs in a rice glutelin gene promoter: minimal *cis*-element requirements for endosperm-specific gene expression. *Plant J.* **23**, 415-421.

145 Wurtzel, E. T., Luo, R. and Yatou, O. 2001. A simple approach to identify the first rice mutants blocked in carotenoid biosynthesis. *J. Exp. Bot.* **52,** 161-166.

146 Wurtzel, E. T. and Vallabhaneni, R. 2009. Timing and biosynthetic potential for carotenoid accumulation in genetically diverse germplasm of maize. *Plant Physiol.* **150**, 562-572.

147 Wyss, A., Wirtz, G., Woggon, W., Brugger, R., Wyss, M., Friedlein, A., Bachmann, H. and Hunzlker, W. 2000. Cloning and expression of beta, beta-carotene 15,15'-dioxygenase. *Biochem. Biophys. Res. Commun.* **271**, 334-336.

148 Xu, N. and Bewley, J. D. 1995. The role of abscisic acid in germination, storage protein synthesis and desiccation tolerance in alfalfa (*Medicago sativa* L.) seeds, as shown by inhibition of its synthesis by fluridone during development. *J. Exp. Bot.* **46**, 687-694.

149 Yamamoto, H. Y. 1985. Xanthophyll cycles. *Methods Enzymol.* **110**, 303-312.

150 Ye, X., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P. and Potrykus, I. 2000. Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science.* **287**, 303-305.

151 Young, A. J., Phillip, D., Ruban, A. V., Horton, P. and Frank, H. A. 1997. The xanthophylls cycle and carotenoid-mediated dissipation of excess excitation energy in photosynthesis. *Pure & Appl. Chem.* **69**, 2125-2130.

152 Zeevaart, J. A. D. and Creelman, R. A. 1988. Metabolism and physiology of abscisic acid. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **39**, 439-473.

153 Zhang, W. and Dubcovsky, J. 2008. Association between allelic variation at the phytoene synthase 1 gene and yellow pigment content in the wheat grain. *Theor. Appl. Genet.* **116**, 635-645.

154 Zile, M. H. 2001. Function of vitamin A in vertebrate embryonic development. *J. Nutr.* **131**, 705-708.